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13. ABSTRACT *(Maximum 200 Words)*
The present research program aims at:
1. Development of a mass spectrometric or fluorescence-based method for retrospective detection of exposure to low doses of sulfur mustard, based on improvement of analysis of the adducted tripeptide cysteine(S-2-hydroxyethylthioethyl)-proline-phenylalanine ((S-HETE)Cys-Pro-Phe) in albumin and of adducts to histidine in hemoglobin and albumin.
2. Development of immunochemical assays for quantitation of adduct levels of sulfur mustard to hemoglobin, albumin and keratin.

The procedure for isolation of albumin from human blood could be substantially shortened by using affinity chromatography. The lowest detectable exposure level was improved by one order of magnitude (1 nM) by work-up of 20 mg amounts of albumin. Furthermore, the use of an internal standard has been worked out. After i.v. administration of sulfur mustard to the marmoset, the specific (S-HETE)Cys-Pro-Phe adduct could be analyzed after pronase digestion of albumin, for at least 28 days after the challenge.
A work-up procedure was developed for rapid isolation of the histidine adducts from amino acid mixtures resulting from acidic hydrolysis of globin or albumin. Derivatization of the histidine adducts with trifluoroacetic acid anhydride for GC-MS analysis resulted in the formation of a tris(trifluoroacetyl) derivative with favorable mass spectrometric properties. For detection with laser-induced fluorescence, various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared.
An immunoslotblot assay was developed for detection of sulfur mustard adducts to keratin. The lower detection limit of the immunoslotblot assay for detection of sulfur mustard adducts to keratin was at 25 fmol adducted sulfur mustard using 0.5 µg keratin/slot, corresponding to an adduct level of 1 sulfur mustard adduct/5 x 10^7 unadducted amino acids. The minimum detectable concentration for in vitro treatment of human callus with sulfur mustard was 0.2 µM.

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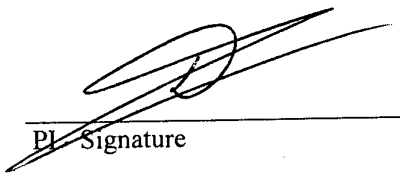

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SUMMARY

The need for retrospective detection procedures for exposure to low levels of chemical warfare agents has been urgently illustrated by the conflicts in the Gulf Area and, especially, in the attempts to clarify the Gulf War Syndrome. Explorative research within the context of our previous grant DAMD17-97-2-7002 has yielded important clues for development of standard operating procedures that satisfy these needs. The present research aims at:

1. development of a mass spectrometric or fluorescence-based method for retrospective detection of exposure to low doses of sulfur mustard, based on improvement of analysis of an adducted tripeptide in albumin and of adducts to histidine in hemoglobin and albumin.
2. development of immunoslotblot assays for quantitation of protein – sulfur mustard adduct levels, using monoclonal antibodies already available from previous research in this field.

The albumin assay is based on our previous finding that upon pronase treatment of sulfur mustard alkylated albumin, a tripeptide cysteine(S-2-hydroxyethylthioethyl)-proline-phenylalanine ((S-HETE)Cys-Pro-Phe) results which has favorable mass spectrometric properties. The procedure for isolation of albumin from human blood could be substantially shortened by performing the incubation with $CaCl_2$ for only 1 h, instead of overnight. Moreover, an affinity chromatography procedure was developed which enables work-up, digestion and mass spectrometric analysis of a plasma sample within 3 hours.
The lowest detectable exposure level was improved by one order of magnitude (1 nM) by work-up of larger amounts of albumin. Furthermore, the use of an internal standard, *i.e.*, albumin isolated from human blood exposed to d_8-sulfur mustard, has been worked out. This will enable quantitative analyses of unknown samples.

Derivatization of (S-HETE)Cys-Pro-Phe with pentafluorobenzyl bromide (PFB-Br) in the presence of KOH in acetonitril afforded the di-PFB derivative, which enabled mass spectrometric detection under Electron Capture Atmospheric Pressure Chemical Ionization (EC APCI) conditions.

With respect to forthcoming animal experiments, in which rats will be exposed to sulfur mustard in order to obtain information about the persistence of the albumin-sulfur mustard adduct, the level of alkylation at the free cysteine residue in rat albumin was determined to be 5%. After pronase treatment of rat albumin, isolated from rat blood exposed to [^{14}C]-labelled sulfur mustard, the tripeptide Cys(HETE)-Pro-Tyr could be analyzed in an analogous way as performed for (S-HETE)Cys-Pro-Phe.
Plasma samples from marmosets which had been challenged (i.v.) with sulfur mustard within the context of our previous cooperative agreement, were worked up. Albumin was isolated and digested with pronase. The tripeptide (S-HETE)Cys-Pro-Phe could still be analyzed after 28 days. Taken into account that the mass spectrometer (Q-TOF) used for these experiments is not the most sensitive one for this kind of analyses, the retrospectivity for this particular adduct is high.

With regard to analysis of the histidine-sulfur mustard adducts (N1- and N3-isomers, *i.e.*, the most abundant amino acid adducts formed after exposure to sulfur mustard), a work-up procedure was developed for rapid isolation of these adducts from amino acid mixtures resulting from acidic hydrolysis of globin or albumin. This procedure consists of an ion chromatography step by using Dowex 50WX8 (Na^+ form). Uncharged amino acids were eluted with aqueous acetic acid. Subsequent elution with diluted NH_4OH afforded highly purified histidine adducts, which could be further derivatized with Fmoc-Cl for electrospray LC tandem MS analysis, or with fluorescent labels for detection with laser induced fluorescence.

From our previous research it appeared that GC-MS based procedures for analysis of the histidine adducts were troublesome, mainly due to the high polarity or thermal instability of the derivatives. We have now found that derivatization with trifluoroacetic anhydride results in the formation of a (tris)trifluoroacetyl derivative with favorable GC-MS properties.
Derivatization of the histidine adducts with pentafluorobenzyl bromide in DMF, in the presence of triethylamine, afforded the corresponding tris-(pentafluorobenzyl) derivatives. ^{1}H-NMR spectroscopy of this derivative indicated that, in addition to the amino- and carboxyl function, the imidazole function had been modified with a pentafluorobenzyl group. Self-evidently, such a derivative cannot be analyzed by GC-MS since it has a permanent positive charge. On the other hand, the derivative exhibited favorable properties for LC-tandem MS analysis.

For detection with laser-induced fluorescence, various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared.

With regard to the development of immunoslotblot assays for detection of sulfur mustard adducts to proteins, several clones are available producing antibodies which show specificity not only for hemoglobin alkylated with sulfur mustard (50 μM) but also for alkylated keratin. An improved screening procedure has been set up for selection of antibodies from clones raised against adducted peptides with amino acid sequences present in human globin.
An immunoslotblot assay was set up for detection of sulfur mustard adducts to keratin. Up to now, the lower detection limit of the immunoslotblot assay for detection of sulfur mustard adducts to keratin was at 25 fmol adducted sulfur mustard using 0.5 μg keratin/slot, corresponding to an adduct level of 1 sulfur mustard adduct/ $5x10^7$ unadducted amino acids. The minimum detectable concentration for *in vitro* treatment of human callus with sulfur mustard was 0.2 μM.

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I INTRODUCTION

The use of chemical warfare agents in the Iran-Iraq war has learned that reliable methods for identification and verification of exposure to chemical warfare agents in alleged casualties were urgently needed, and not available at that time. Furthermore, experience with the casualties in the Iran-Iraq war and with other incidents learned that biopsies or autopsies of alleged victims often become available several days or even weeks after alleged exposure. Recently, the need for retrospective detection of exposure and, even more demanding, of low level exposure has been vividly illustrated in the attempts to clarify the causes of the so-called "Persian Gulf War Syndrome". Quantitation of low level exposure provides an indispensable basis to study the subtle toxic effects of such exposures. Moreover, the application of reliable procedures to exclude that even trace exposure to chemical agents has occurred will contribute to combat readiness of the soldier.

Within the framework of previous grants (DAMD17-88-Z-8022, DAMD17-92-V-2005 and DAMD17-97-2-7002) we have worked on the development of methods for diagnosis and dosimetry of exposure to sulfur mustard (*1-16*). Our approach is based upon the development of monoclonal antibodies against adducts of sulfur mustard with DNA and proteins for use in a variety of immunochemical assays and upon the development of procedures for mass spectrometric analysis of the adducts. The immunochemical assays can be performed on small samples, are highly sensitive, and can be applied "on site" when properly developed. Results obtained from the GC-MS or LC-MS-MS analyses will confirm the immunochemical results and will provide information on the structure of the adducts. In this way, it can be established whether casualties have indeed been exposed to sulfur mustard, whereas dosimetry of the exposure will be a starting point for proper treatment of the intoxication.
The main advantage of detection of adducts of sulfur mustard in proteins over those to DNA is the expected much longer half-life of protein adducts (*17*). Whereas in DNA of human skin most of N7-(2-hydroxyethylthioethyl)-guanine (N7-HETE-Gua)[1] has been removed two days after *in vivo* exposure (*18*), it is expected that adducts to proteins have life-spans varying from several weeks to months. Consequently, the retrospectivity of the diagnosis in protein adducts is superior to that in DNA. Moreover, detection is supposedly also more sensitive in case of single, protracted, and intermittent exposure to sulfur mustard at low concentrations, since the protein adducts will accumulate.
Within the framework of our previous grant DAMD17-97-2-7002 we have drafted standard operating procedures (SOPs) for the immunoslotblot (ISB) assays of sulfur mustard adduct to DNA and for the GC-NCI/MS determination of the sulfur mustard adduct to N-terminal valine in human hemoglobin. The development of the SOPs exposed some problems. For instance, with regard to the assay for GC-NCI/MS determination of the sulfur mustard to N-terminal valine in human hemoglobin, we were not able to further decrease the lowest detectable exposure level, *i.e.*, 100 nM. Nevertheless, these SOPs represent the first practically useful methods that have been validated for diagnosis of exposure to sulfur mustard which can be applied in a sophisticated field laboratory. Although the lowest detectable exposure levels of these procedures appeared to be sufficient to prove mild exposure to sulfur mustard of Iranian soldiers in blood samples taken 3 weeks after exposure (*8*), it is self-evident that further lowering of these detection limits is needed when even lower exposure levels should be firmly established. The need for research on such detection of low level exposure and effects thereof has been formulated as a high priority research goal by the Department of Defense (1999).
Explorative research within the context of our previous grant DAMD17-97-2-7002 has yielded important clues for development of new SOPs that satisfy the abovementioned needs. Firstly, we have lowered the minimum detectable concentration of sulfur mustard in human blood by at least one order of magnitude by mass spectrometric analysis of adducted peptides from enzymatically digested albumin (*11*). Secondly, we have found that, as in the case of

[1] HETE: 2-(hydroxyethyl)thioethyl

hemoglobin, adducts to histidine are by far the most abundant adducts in albumin. Thirdly, we have recently succeeded to raise monoclonal antibodies against sulfur mustard adducts to hemoglobin, albumin and keratins. The latter opens the possibility to detect *in vivo* skin exposure to sulfur mustard in a non-invasive way under operational conditions, *e.g.*, by means of immunofluorescence techniques. If these qualitative immunoassays indicate that exposure to sulfur mustard has occurred, adduct levels have to be quantified in order to estimate the extent of sulfur mustard injury.

Consequently, we decided to continue our investigations on four topics, within the framework of the current Cooperative Agreement DAMD17-02-2-0012:

1. further development of the mass spectrometric analysis of the tripeptide (S-HETE)Cys-Pro-Phe, resulting from pronase digestion of albumin alkylated by sulfur mustard.
2. perform further research on analysis of the most abundant adduct formed after exposure of proteins to sulfur mustard, *i.e.*, N1/N3-HETE-histidine.
3. develop the most suitable procedure into a SOP.
4. develop immunochemical assays in order to quantify levels of sulfur mustard adducts to proteins.

II MATERIALS AND INSTRUMENTATION

II.1 Materials

WARNING: Sulfur mustard is a primary carcinogenic, vesicant, and cytotoxic agent. This compound should be handled only in fume cupboards by experienced personnel.

Technical grade sulfur mustard was purified by fractional distillation in a cracking tube column (Fischer, Meckenheim, Germany) to a gas chromatographic purity exceeding 99.5%. The following compounds were synthesized as described previously: Nα-Fmoc-(N1/N3-HETE)histidine (*9*), [^{14}C]sulfur mustard (*10*) and sulfur mustard-d_8 (*4*)

The following commercially available products were used:
Fluorescein-5-isothiocyanate (FITC 'isomer I'), 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) and Oregon Green 488-X, succinimidyl ester 6-isomer (Molecular Probes Europe BV, Leiden, The Netherlands). Diethanolamine, acetonitrile (Baker Chemicals, Deventer, The Netherlands); human serum albumin, pentafluorobenzyl bromide (HSA), (Fluka, Buchs, Switzerland); dl-dithiothreitol (DTT), iodoacetic acid sodium salt, TPCK trypsin, bovine serum albumin, human hemoglobin, pronase Type XIV from Streptomyces Griseus (E.C. 3.4.24.31), tween 20 (polyoxyethylenesorbitan monolaurate) (Sigma Chemical Co., St. Louis, MO, U.S.A.); immobilized TPCK-trypsin (14 units/ml gel) heptafluorobutyrylimidazole (Pierce, Rockford, IL, U.S.A.); 9-fluorenylmethylchloroformate (Fmoc-Cl), β-mercaptoethanol, Tris.HCl, EDTA (Janssen, Beerse, Belgium); benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; PyBOP (Novabiochem); trifluoroacetic acid, thiodiglycol (Aldrich, Brussels, Belgium); fetal calf serum (FCS; LCT Diagnostics BV, Alkmaar, NL); goat-anti-mouse-Ig-alkaline phosphatase, goat anti-mouse-IgG-alkaline phosphatase (KPL, Gaithersburg, USA); microtiter plates (96 wells; polystyrene 'high binding'), microtiter plates (96-wells culture plates) (Costar, Badhoevedorp, The Netherlands); 4-methylumbelliferyl phosphate (MUP), dispase, RNAse T1, protease inhibitor cocktail mini tablets, (Boehringer, Mannheim, Germany); rabbit-anti-mouse-Ig-horse radish peroxidase (Dakopatts, Glostrup, Denmark); FITC-labeled 'goat-anti-mouse' (Southern Biotechnology Associates, Birmingham, AL), RPMI-1640 medium (Gibco BRL, Breda, The Netherlands); skimmed milk powder, less than 1% fat, (Campina, Eindhoven, The Netherlands). Carbosorb and Permablend scintillation cocktail were obtained from Canberra Packard (Tilburg, The Netherlands).
Slyde-A-Lyzer cassettes were obtained from Pierce. Centrex UF-2 10 kDa filters were obtained from Schleicher & Schuell (Dassel, Germany). SepPak Florisil and SepPak C-18 cartridges were obtained from Waters (Bedford, MA). Centrex UF-2 (3 or 10 kDa molecular weight cut-off) centrifugal ultrafilters were procured from Schleicher & Schuell (Keene, NH). Ultrafree (100 kD molecular weight cut-off; 15 ml) centrifugal ultrafilters were obtained from Millipore (Bedford, MA). Albumin affinity chromatography was carried out on HiTrap Blue HP columns (1 ml; Amersham Biosciences, Uppsala, Sweden). Desalting of albumin fractions was carried out on PD-10 columns containing Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden). Dowex 50WX8 was obtained from Fluka.
Venous blood of human volunteers (10 ml, with consent of the donor and approval of the TNO Medical Ethical Committee) was collected in evacuated glass tubes, containing Na_2EDTA (15 mg).
Human callus was obtained from chiropodists. Human skin resulting from cosmetic surgery was obtained from a local hospital with consent of the patient and approval of the TNO Medical Ethical Committee.

II.2 Instrumentation

UV absorbance and UV spectra were recorded on a UV/VIS Spectrometer, Lambda 40 (Perkin Elmer, Breda, The Netherlands).
Gel filtration on Sephadex G-75 (Pharmacia) was performed with a P-1 pump, GP-250 gradient programmer, Frac-100 fraction collector, a UV-1 optical unit (254 nm) and a UV-1 control unit (Pharmacia).
Microtiter plates were washed using the Skanwasher 300 (Skatron Instruments, Norway; Costar). The fluorescence on microtiter plates (excitation at 355 nm; emission at 480 nm) was recorded with a Cytofluor II (PerSeptive Biosystems, Framingham, MA).
Immunoslotblot assays were carried out with Schleicher & Schuell minifold S (6 mm^2 slots) and nitrocellulose filters (pore size 0.1 µm; Schleicher and Schuell). Protein was immobilized by UV-crosslinking with a GS Gene Linker UV chamber (Bio-Rad Laboratories, The Netherlands). An Enhanced Chemiluminescence Blotting Detection System (Boehringer) was used for the detection of peroxidase activity. The chemiluminescence was recorded with a 1450 MicroBeta Trilux Luminescence Counter (EG & G Wallac, Breda, The Netherlands).

Liquid chromatography experiments were run on an ÅKTA explorer chromatography system (Amersham Pharmacia, Uppsala, Sweden). Columns used were a Pep RPC 5/5 column (Pharmacia, Uppsala, Sweden), a Zorbax SB C-18 column (4.6 mm x 150mm; 5 µm, Zorbax, Mac-Mod Analytical, Chadds Ford, PA, USA) and a Source 15 RPC column (Amersham Pharmacia, Uppsala, Sweden).
HPLC with radiometric detection was performed using a Gilson (Villers-le-Bel, France) HPLC system consisting of a 305 master pump, a 306 slave pump, an 805 manometric module and an 811C dynamic mixer. The mobile phase consisted of a linear gradient (0'-20') of 0.1% (v/v) trifluoroacetic acid (TFA) in water to 48% (v/v) acetonitrile and 52% (v/v) water with 0.1% (v/v) TFA. The LC flow was 1 ml/min. The eluate was monitored at 214 nm with a Spectroflow 757 UV detector (Applied Biosystems, Ramsey, NJ) and with a radiometric detector (Radiomatic, model Flo-one Beta series A 500, Meriden, CT) with Ultima-Flo (Packard, Meriden, CT) as scintillation cocktail. Liquid scintillation countings were performed with a A2500 TR scintillation counter (Packard) with Hionic Fluor (Packard) as scintillation cocktail.
LC/electrospray tandem mass spectrometric experiments were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 µl injection loop mounted and a PepMap C_{18} (LC Packings) or Vydac C18 column (both 15 cm x 300 µm I.D., 3 µm particles). A gradient of eluents A (H_2O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approximately 6 µl/min through the column and into the electrospray MS interface. MS/MS product ion spectra were recorded using a cone voltage between 25 and 40 V and a collision energy between 30 and 35 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).
Other LC/electrospray tandem MS spectra were recorded on a VG Quattro II triple quadrupole mass spectrometer (Micromass, Altrincham, U.K.). The analyses were carried out with multiple reaction monitoring at a dwell time of 2 s, unless stated otherwise. Operating conditions were: capillary voltage 3.6 kV, cone voltage 25 V, collision energy 15 eV, gas (argon) cell pressure 0.3 Pa, and source temperature 120 °C.
APCI-MS was performed on a TSQ7000 (Finnigan) at TNO Food and Nutrition Institute (Zeist, The Netherlands).
Fourier Transform – Ion Cyclotron Resonance (FT-ICR) MS was performed on a 11.5-T instrument (Environmental Molecular Sciences Laboratory, Richland, Washington). Sector-

ion trap mass spectrometry was performed on a BE ion trap MAT900 (Thermoquest, Bremen, Germany).

GC-NCI/MS analyses were carried out with a HP 5973 mass selective detector connected to a HP 6890 GC system with an HP 7673 autoinjector, using pulsed splitless injection. The column used was a Restek RTX-5SilMS capillary column (length 30 m, i.d. 0.25 mm, film thickness 1 µm). The oven of the chromatograph was kept at 100 °C for 1.5 min, the temperature was then progammed at 25 °C/min to 270 °C. Source temperature MS: 160 °C. Injection volume was 1 µl (containing about 1% of the total sample).

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian (Palo Alto, CA, U.S.A.) VXR 400S spectrometer operating at 400.0 MHz and 100.6 MHz, respectively. Chemical shifts are given in ppm relative to tetramethyl silane. The solvent signals at 2.525 ppm (residual Me_2SO-d_5 in Me_2SO-d_6) or 7.260 ppm (residual $CHCl_3$ in $CDCl_3$) served as a reference for ^{1}H NMR spectroscopy, whereas the solvent signals at 39.6 ppm (Me_2SO-d_6) or 77.1 ppm ($CDCl_3$) were used as a reference for ^{13}C NMR spectroscopy.

Radioactivity countings were performed on a Packard Tri-Carb series Minaxi (Downers Grove, IL, U.S.A.) or a Packard Mark III liquid scintillation spectrometer with Picofluor 30 (Packard) as a scintillation cocktail.

III EXPERIMENTAL PROCEDURES

III.1 Development of assays for analysis of sulfur mustard adducts to proteins as Standard Operating Procedures: mass spectrometric analysis of an alkylated tripeptide in albumin

III.1.1 Incubation of human blood with sulfur mustard, [^{14}C]sulfur mustard or sulfur mustard-d_8

A 1 M solution of sulfur mustard, [^{14}C]sulfur mustard (sp. act. 15 mCi/mmol) or sulfur mustard-d_8 in CH_3CN was prepared. Subsequently, the required dilution in CH_3CN was prepared and a well-defined amount was added to human blood (2-10 mL), resulting in a 1% end concentration of CH_3CN. After incubation for 2 h at 37 °C, plasma and erythrocytes were separated by centrifugation at 3,000 rpm.

III.1.2 Isolation of albumin from plasma by precipitation

Albumin was isolated from human plasma according to a procedure described by Bechtold *et al.* (*20*). Thus, shortly, whole blood was collected into an EDTA-containing vacutainer and separated into red blood cells and plasma. To the plasma an equal volume of 0.5 M $CaCl_2$ was added. The mixture was incubated at room temperature overnight, or for 1 h in the shortened version of the procedure and then centrifuged at 900*g* for 20 min. To the supernatant were added 4 volumes of 0.9% saline. Nine volumes of an acid/alcohol mixture (made by adding 1 ml 12 M HCl to 600 ml ethanol) were added dropwise to the supernatant. The mixture was incubated at 37 °C for 30 min and then centrifuged at 650*g* for 5 min. To the supernatant was added a volume of 0.2 M sodium acetate in 95% ethanol equal to 1/10 the total volume of the supernatant. After 15 min the mixture was centrifuged at 650*g* for 5 min, the supernatant discarded, and the albumin pellet washed with acetone. The mixture was centrifuged at 650*g* for 5 min and the supernatant discarded. The pellet was then washed in diethyl ether, centrifuged and allowed to dry overnight. Yields: 50-60 mg/ml plasma. Analysis with SDS PAGE showed coelution with commercially available human serum albumin.

III.1.3 Isolation of albumin from human plasma, using affinity chromatography

Plasma (1 mL) was applied on a HiTrap™ Blue HP (prepacked with Blue Sepharose High Performance, with Cibacron Blue F3G-A as the ligand; 1 mL) affinity column that was incorporated into an FPLC system, after conditioning with buffer A (50 mM KH_2PO_4, pH 7; 10 mL). The column was eluted with buffer A (7 mL; 1 mL/min). A large peak was visible at 280 nm, corresponding with material having no affinity to the column material. Subsequently, the column was eluted with buffer B (50 mM KH_2PO_4, 1.5 M KCl, pH 7; 7 mL; 1 mL/min). UV positive (280 nm) material was collected (total volume 2.5 mL). The HiTrap column was regenerated by washing with buffer A (14 mL).
Subsequently, a PD-10 column (containing 10 mL of Sephadex G 25 material) was equilibrated with 50 mM NH_4HCO_3 (25 mL). The albumin fraction, collected from the HiTrap Blue HP column (2.5 mL), was applied to the column, and the column was eluted with aqueous NH_4HCO_3 (50 mM; 0.5 mL). Next, the column was further eluted with aqueous NH_4HCO_3 (50 mM; 2.5 mL) and the eluate was collected.

III.1.4 Pronase digestion of large amounts of albumin and LC-tandem MS analysis of (S-HETE)Cys-Pro-Phe

To a suspension of albumin (20 mg) in aqueous NH_4HCO_3 (50 mM; 5 ml) was added a solution of pronase (10 mg/ml; 660 µl) in aqueous NH_4HCO_3 (50 mM). After incubation for

2.5 h at 37 °C, the mixture was filtrated through a filter with a cut-off of 3 kDa with centrifugation at 4,000*g*, in order to remove the enzyme. Sep-Pak C18 clean-up of the sample was performed as follows. A Sep-pak C18 cartridge (model Classic) was rinsed with MeOH (5 ml) followed by 0.1% TFA/H_2O (5 ml). The filtered pronase digest was applied to the cartridge. The cartridge was rinsed consecutively with 0.1% TFA/H_2O (2 ml), 0.1% TFA/10% CH_3CN (2 ml), 0.1% TFA/20% CH_3CN (2 ml) and finally with 0.1% TFA/40% CH_3CN (2 ml). The 40% CH_3CN eluate was collected, concentrated and redissolved in H_2O (50 µl). The sample was now ready for LC-MS analysis. The tripeptide (S-HETE)Cys-Pro-Phe is determined by multiple reaction monitoring (MRM) of MH^+ (*m/z* 470) → *m/z* 105 which corresponds with a fragment of thiodiglycol. Operation conditions were: cone voltage 30 - 35 V, collision energy 20 eV and argon pressure $3\text{-}4.10^{-3}$ mB. The injection volume was 40 µl. The LC-system comprised a microcolumn with Lichrosorb RP18 material (length 0.35 m, i.d. 0.32 mm). Gradient elution using H_2O/CH_3CN 95/5 with 0.2% HCOOH and H_2O/CH_3CN 2/8 with 0.2% HCOOH as eluent A and B, respectively, was performed as follows.
0-5 min: flow 0.1 ml/min, 100% eluent A; 5-25 min: flow 0.6 ml/min, 100% A to 70% A; 25-45 min: flow 0.6 ml/min, 70% A to 0 % A. Flow rates were reduced by means of an LC Packings splitter (Amsterdam, The Netherlands) which was placed before the injection valve: 0-5 min, 2 → 10 µl/min and subsequently 10 µl/min.

III.1.5 Digestion of albumin obtained after affinity chromatography, with addition of internal standard

Plasma samples (1 mL), isolated from blood exposed to different concentrations of sulfur mustard were spiked with plasma (50 µL), isolated from blood exposed to 100 µM d_8-sulfur mustard. Next, these samples were applied to a HiTrap Blue Sepharose column and desalted on a PD-10 column, as described above.
Pronase digestion of a sample (0.25 mL) of the purified albumin fraction (diluted with aqueous NH_4HCO_3; 50 mM; 0.5 mL), followed by LC/MS/MS analysis, showed a lineair relationship between exposure level and peak ratio.

III.1.6 Synthesis of (S-d_8-HETE)-Cys-Pro-Phe

To a solution of Cys-Pro-Phe (1.8 mg; 5 µmol) in CH_3CN/H_2O, 1/1, v/v) was added 5% $NaHCO_3$ (1.25 mL). Subsequently, a solution of d_8-sulfur mustard in CH_3CN (80 mg/ml; 25 µL) was added. The solution was stirred for 2 h at room temperature. HPLC analysis showed conversion into one major product. Subsequently, the solution was neutralized with 1 M aqueous HCl and the product was purified by means of reversed phase HPLC. Mass spectrometric analysis showed the expected molecular mass; no d_0-derivative could be detected. Yield: 1.8 mg (3.7 µmol; 75%) of a colourless oil.

III.1.7 Derivatization of (S-HETE)-Cys-Pro-Phe for LC tandem MS analysis

Derivatization was accomplished according to Singh et al (*19*). Shortly, to a solution of the adduct (5 µg) in acetonitrile (50 µl) was added acetonitrile/pentafluorobenzyl bromide (19/1, v/v; 50 µl) and a solution of KOH in EtOH (8/1000, w/v; 50 µl). The mixture was heated at 60 °C for 30 min. Subsequently, the mixture was concentrated and analyzed by LC/electrospray tandem MS on a TSQ7000 instrument.

III.1.8 Synthesis of (S-HETE)Cys-Pro-Tyr

This compound was synthesized on solid phase, in an analogous way as (S-HETE)-Cys-Pro-Phe, using N-Fmoc-(S-HETE)Cys (*11*). Tandem mass spectrometric data: m/z 486.2 (MH^+),

469.2 ($MH^+ - NH_3$), 279.2 (Pro-Tyr), 137.0 ($^+S\text{-}CH_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH_2\text{-}OH$), 105.0 ($^+CH_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH_2\text{-}OH$).

III.2 Development of assays for analysis of sulfur mustard adducts to proteins as Standard Operating Procedures: mass spectrometric and fluorescence-based analysis of histidine adducts

III.2.1 Isolation of globin from human blood

Globin was isolated from human blood samples as described earlier (*4*). The red blood cells were washed four times with saline and lysed with water. After 30 min in ice/water, they were centrifuged for 30 min at 25,000*g* (4 °C). The supernatant was poured into a stirred mixture of concentrated HCl/acetone (1/100, v/v) at -20 °C. After decanting the supernatant, the precipitate was washed with concentrated HCl/acetone (1/100, v/v), acetone and ether, and dried. For some experiments, the crude globin was purified by chromatography on a G-25 Sephadex column, using 0.1 M formic acid, 6 M urea and 50 mM dithiothreithol as an eluent. UV-positive fractions were pooled and dialyzed three times against a 1 mM phosphate buffer, pH 7. Finally, the globin was dialyzed against water for 2 h and lyophilized to give a white fluffy compound.

III.2.2 Acidic hydrolysis of globin

Globin (20 mg), isolated from human blood, was hydrolyzed in constant boiling HCl (6 M, 110 °C, 16 h) in hydrolysis tubes. Next, the clear solution was evaporated to dryness and coevaporated several times with water.

III.2.3 Hydrazinolysis of globin

Globin (26 mg), isolated from human blood which had been exposed to 10 µM sulfur mustard, was dissolved in hydrazine monohydrate (4.5 mL) and heated at 100 °C overnight. The resulting pink solution was concentrated under reduced pressure. Analysis with LC-MS showed the presence of both HETE-His-N_2H_4 (MH^+ 274) and HETE-His-OH (MH^+ 260).

III.2.4 Procedure for isolation of histidine adducts by means of cation-exchange chromatography, derivatization with Fmoc-Cl, and Sep-Pak C18 clean-up

A disposable syringe (10 mL size) was filled with Dowex WX8 resin (Na^+ form; 3 mL) and the resin was washed with aqueous acetic acid (15 mL; pH 5.5). A solution of globin acidic hydrolysate (from 3 mg of globin; coevaporated several times with water, to evaporate excess HCl) in aqueous acetic acid (1 mL; pH 5.5) was applied to the column. The column was washed with 15 mL aqueous acetic acid (pH 5.5) in order to remove uncharged and weakly basic amino acids. Subsequently, the column was washed with water (10 mL) to remove acetic acid. Finally, N1/N3-HETE-histidine and other basic amino acids were removed from the column using aqueous NH_4OH (15 mL, pH 10.5). The latter eluate was evaporated to dryness. Fmoc derivatization and Seppak C18 purification were performed as described earlier (*9*).

III.2.5 Derivatization procedures for GC-MS analysis of histidine adducts

Derivatization of N1/N3-HETE-histidine with trifluoroacetic anhydride and GC-MS analysis of the derivative

N1/N3 HETE-histidine (1.0-10 mg) was dissolved in 2 M HCl in methanol (2 ml) and heated at 80 °C for 1h. After evaporization under reduced pressure, ethyl acetate (1 ml) and trifluoroacetic anhydride (0.5 ml) were added and the solution was heated at 110 °C for 15

min-90 min and evaporated under reduced pressure. GC-MS analysis showed that a derivative with 3 trifluoroacetyl groups had been formed.
GC-NICI-MS: m/z 561 (HETE-His-OMe.3 TFA). GC-EI-MS: 141 (CF_3-C(O)-O-$C_2H_4^+$), 201 (CF_3-C(O)-O-C_2H_4-S-$C_2H_4^+$)
detection limit: 3 pg

Derivatization of HETE-histidine with heptafluorobutyric anhydride and GC-MS analysis of the derivative
N3-HETE-histidine (1.0 mg) was dissolved in 2 M HCl in methanol (2 ml) and heated at 80 °C for 1h. After evaporization under reduced pressure ethyl acetate (1 ml) and heptafluorobutyric anhydride (0.5 ml) were added and the solution was heated at 110 °C for 15 min and evaporated under reduced pressure.
GC-NICI-MS: m/z 665 (HETE-His-OMe.2 HFB)
note: N1 HETE-histidine was also derivatisized with HFBA, but the desired adduct could not be detected.

Derivatization of N3-HETE-histidine with PFPA and GC-MS analysis of the derivative
N3-HETE-histidine (1.0 mg) was dissolved in 2 M HCl in methanol (2 ml) and heated at 80 °C for 1h. After evaporization under reduced pressure ethyl acetate (1 ml) and pentafluoropropionic anhydride (0.5 ml) were added and the solution was heated at 110 °C for 15 min and evaporated under reduced pressure.
GC-NICI-MS: m/z 565 (HETE-His-OMe.2 PFP).

Derivatization of N1-HETE-histidine methyl ester with hydrazine
Boc-N1-HETE (OtBu)-His-OMe (18.3 mg) was dissolved in TFA/H_2O (95/5, v/v; 2 ml) and left at room temperature for 2 h. The solution was concentrated under reduced pressure and the residue was coevaporated with ethanol (2x 2 ml). The residue was dissolved in isopropanol (210 µl) and hydrazine monohydrate (36 µl) was added. After standing at room temperature overnight the solution was concentrated under reduced pressure and the residue was coevaporated with methanol (2x 2 ml). LC-MS: *m/z* (MH^+) 274.1.

Derivatization of N1-HETE-histidine hydrazide
N1-HETE-histidine hydrazide was dissolved in THF (1 mg/ml). To 200 µl of this solution trifluoroacetic anhydride, pentafluoropropionic anhydride or N-methyl-bis-trifluoroacetamide (60 ul) was added and the mixture was heated at 110 °C for 15 min. The mixtures were concentrated under reduced pressure. MS analysis showed that HETE-histidine-hydrazide was very reactive towards THF; only THF adducts could be detected.

III.2.6 Fluorescence derivatization of histidine adducts

FITC derivatives of N1/N3-(2-hydroxyethylthioethyl)histidine
To an aqueous solution of N1- or N3-(2-hydroxyethylthioethyl)histidine (0.5 ml; 3.6 mM) was added aqueous Na_2CO_3 (0.5 ml; 0.2 M) and a solution of fluorescein-5-isothiocyanate (FITC 'isomer I') in ethanol (1 ml; 2.5 mM). The mixture was vigorously vortexed and left at room temperature under the exclusion of light. A reaction mixture without the histidine derivative was taken as control. After 4 h, FPLC analysis demonstrated the presence of a strong UV-absorbing compound for each isomer, which was not present in the chromatogram obtained for the control sample. The compounds were isolated by semi-preparative FPLC, affording yellow-orange solids. Electrospray MS: *m/z* 649 (MH^+), 325 (MH_2^{2+}) for both derivatives.
The N1- and N3-(2-hydroxyethylthioethyl)histidine FITC derivatives had slightly different retention times.

In aqueous solution, the compounds slowly (16 h at room temperature) rearranged into compounds with *m/z* 631 (MH^+; probably the corresponding thiohydantoins), which had a slightly different retention time.

CBQCA derivatives of N1/N3-(2-hydroxyethylthioethyl)histidine
To an aqueous solution of N1- or N3-(2-hydroxyethylthioethyl)histidine (0.65 ml; 2.9 mM) was added a solution of 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) in methanol/0.2 M aqueous KOH, 80/1, v/v (0.65 ml; 10 mM) and an aqueous KCN solution (1.3 ml; 20 mM). The mixture was vigorously vortexed and left at room temperature under the exclusion of light. A reaction mixture without the histidine derivative was taken as control. After 3 h, FPLC analysis demonstrated the presence of a strong UV-absorbing compound for each isomer, which was not present in the chromatogram obtained for the control sample. The compounds were isolated by semi-preparative FPLC, affording dark pink solids. Yield for the N3 adduct: 0.4 mg; 0.7 µmol; 38% based on histidine derivative. Electrospray MS: *m/z* 556 (MH^+), 452 (MH^+ - $HOCH_2CH_2SCH_2CH_2^+$).

Oregon Green 488-X derivatives of N1/N3-(2-hydroxyethylthioethyl)histidine
To a solution of N1- or N3-(2-hydroxyethylthioethyl)histidine (0.9 µmol) in aqueous $NaHCO_3$ (2 ml; 0.2 M) was added a solution of Oregon Green 488-X, succinimidyl ester 6-isomer in ethanol (1 ml; 2 mM). The mixture was vigorously vortexed and left at room temperature under the exclusion of light. A reaction mixture without the histidine derivative was taken as control. After 4 h, FPLC analysis demonstrated the presence of a strong UV-absorbing compound for each isomer, which was not present in the chromatogram obtained for the control sample. The compounds were isolated by semi-preparative FPLC, affording orange solids. Electrospray MS: *m/z* 789 (MNa^+), 767 (MH^+), 384 (MH_2^{2+}).

III.3 Development of immunoslotblot assays for quantitative analysis of sulfur mustard adducts to proteins

III.3.1 Exposure of human skin to sulfur mustard vapor

The device as represented in Figure 1 was used for exposure of human skin. The estimated sulfur mustard concentration at 28 °C close to the skin was 1100 $mg.m^{-3}$.

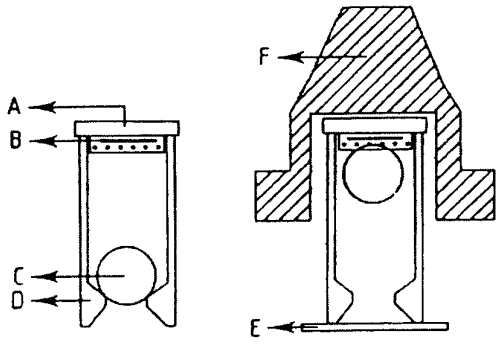


Figure 1. Schematic diagram of the device for skin exposure to air saturated with sulfur mustard vapor. At the inner side of the plastic cap (A) a piece of filter paper (B) was placed, onto which 3 µl of liquid sulfur mustard was applied. The bottom side of the glass cylinder was closed by a stainless steel ball (C). When the air in the cylinder (a volume of ca. 2 ml) had become saturated with sulfur mustard vapor (at 28 °C: ca. 1100 mg/m^3), the cylinder was placed onto the skin (E) and the ball was lifted with a magnet (F) for the desired period of time.

In addition, pieces of skin (0.5 × 0.5 cm) were covered with a solution of sulfur mustard (1 ml of 0, 50 or 100 µM) in PBS containing 1% acetonitrile, for 30 min.

III.3.2 Preparation of skin cryostat sections

After exposure, a piece of the skin was cut from the central part of the treated area and fixed in methanol/acetic acid (3/1 v/v, 1.5 h at 4 °C), rehydrated by incubation overnight in 70% ethanol at 4 °C, followed by incubation in 5% sucrose at 4 °C for 1.5 h. Next, the pieces were stretched between microscope slides and stored at –20 °C.
Alternatively, the piece of skin was immediately stretched between microscope slides, without fixation, and stored at -20 °C.
For the preparation of cryostat sections, a small piece of skin was embedded in Tissue Tek (O.C.T. compound, Miles Inc., Elkhart, USA). Subsequently, cryostat sections (5 mm thickness) were prepared at -35 °C with a cryostat microtome (2800 Frigocut, Rechert-Jung, Leica, Rijswijk, The Netherlands) on slides precoated with a solution of 3-aminopropyl triethoxysilane (2% in acetone). The slides were stored at room temperature. In the case of non-fixated skin, the cross-sections were fixated with 70% ethanol, washed with TBS (20 mM Tris HCl, 150 mM NaCl, pH 7.4) and stored at room remperature.

III.3.3 Detection of keratin adducts; isolation of keratin from human callus

Human callus (100 mg) was soaked in Tris.HCl buffer (5 ml, 20 mM, pH 7.4) overnight. After centrifugation (30 min, 400 rpm) the residue was stirred in a buffer (5 ml; pH 7.4) containing Tris.HCl (20 mM) and urea (8 M). After centrifugation (30 min, 400 rpm), the residue was extracted with a buffer (5 ml; pH 7.4) containing Tris.HCl (20 mM), urea (8 M), and β-mercaptoethanol (0.1 M).
The crude keratin was purified on a G 75 column (100 × 2 cm) with a buffer (pH 7.6) containing SDS (0.5%), Tris.HCl (10 mM) and DTT (10 mM); flow, 0.25 ml/min. Appropriate fractions were collected and dialysed against water. The remaining solution was lyophilized. Representative yield: 20 mg keratin/100 mg callus.

III.3.4 Exposure of human callus to sulfur mustard

To a suspension of human callus (70-100 mg) in 0.9% NaCl (100 µl) was added a solution of an appropriate concentration of sulfur mustard in isopropanol (100 µl). The mixture was incubated for 6 h at 37°C. Isolation of keratin was performed as described in Subsection III.3.3.

III.3.5 Exposure of human skin to saturated sulfur mustard vapor and extraction of epidermal keratins

Human skin samples (9 × 0.25 cm^2) were exposed to saturated sulfur mustard vapor (0, 2, 4 or 8 min) according to the method described in Subsection III.3.6. After the exposure, the epidermis was separated from the dermis by heat shock (2 min at 60 °C, followed by 5 min at 0 °C). The epidermis was cut into 3 or 4 pieces, and transferred into a 4 ml vial. Next, low salt buffer (10 mM Tris/150 mM NaCl/3 mM EDTA/0.1% N-P40; pH 7.4; 3 ml), containing a protease inhibitors cocktail (1 tablet/12 ml buffer), was added. The mixture was shaken for 1 h at 0 °C. The epidermis was sedimented, the upper layer was discarded and replaced by high salt buffer (10 mM Tris/150 mM NaCl/1.5 M KCl/3 mM EDTA/0.1% N-P40; pH 7.4; 3 ml) containing the protease inhibitors cocktail (1 tablet/12 ml buffer). The mixture was shaken for 1 h at 0 °C. Next, the epidermis was sedimented; the upper layer was discarded and replaced by washing buffer (10 mM Tris/150 mM NaCl/3 mM EDTA; pH 7.4; 3 ml) containing the protease inhibitors cocktail (1 tablet/12 ml buffer). After shaking for 30 min at 4 °C, the

epidermis was sedimented. The liquid layer was discarded and the epidermis was extracted with lysis buffer (20 mM Tris/1 mM EDTA/2% SDS/1 mM DTT; pH 7.4, 2 ml), containing protease inhibitors, under gentle shaking overnight at room temperature. The mixture was centrifuged at 5,000*g* for 5 min and the supernatant containing the keratins (0.87 mg/ml) was stored at –70 °C. Before treatment with 0.5 M NaOH, the keratin solutions were dialyzed against H_2O in a Slyde-A-Lyzer cassette (size: 0.1-0.5 ml).

III.3.6 Preparation of skin cryostat section and immunofluorescence microscopy

The preparation of skin cryostat sections has been described in Subsection III.3.2. Immunofluorescence microscopy of sulfur mustard-keratin adducts in skin sections was performed analogously to the procedure described for detection of N7-HETE-Gua (*12*). Briefly, the following procedure was applied after fixation of the skin section with 70% ethanol on aminoalkylsilane-precoated slides and washing with TBS:

- precoating with TBS + 5% milkpowder (30 min at room temperature);
- treatment with antibody specific for sulfur mustard-exposed keratin; supernatants of up to 32 selected monoclonal antibodies in a 1:1 dilution in TBS containing 0.05% Tween 20 and 0.5% gelatin (overnight at 4 °C);
- treatment with a second antibody, FITC-labeled 'goat-anti-mouse', 75-fold diluted in TBS containing 0.05% Tween 20 and 0.5% gelatin (2 h at 37 °C);
- counterstaining with propidium iodide (100 ng/ml, 10 min at room temperature).

Twin images were obtained with a LSM-41 laser scanning microscope. The fluorescence of the FITC group above the stratum corneum and of the propidium iodide were measured consecutively to visualize the presence of sulfur mustard-keratin adducts in the stratum corneum and the DNA in the nuclei. The fluorescein staining was used to determine the presence of sulfur mustard-keratin. Adduct levels were estimated from the brightness of the fluorescence above the stratum corneum. The second image, from the propidium iodide staining, served to localize nuclei on the image.

III.3.7 Immunoassays (ELISA) with hybridoma-supernatants

As described under 'Results' the screening of hybridoma supernatants was subject to detailed investigation. So far the following procedures yielded optimal results:
The hybridoma-supernatants were tested in a direct ELISA against globin isolated from human blood treated with sulfur mustard (0, 50, 100, 500 µM). The ELISA was performed as follows. Polystyrene 'high binding' 96-well microtiter plates were coated with adducted and non-adducted globin dissolved in PBS to a final concentration of 10-12.5 µg/ml. Of these dilutions 50 µl was added per well and incubated 1 h at room temperature. The plates were washed three times with PBS. Next, the plates were incubated with PBS containing 2% FCS for 30 min at room temperature. After emptying the plates, the hybridoma supernatants were added, diluted 100-500 times in PBS with 0.05% Tween 20 and 1% FCS. Of these dilutions 50 µl was added per well and incubated for 30 min at room temperature. After washing with PBS containing 0.05% Tween 20 (three times), the second antibody, viz., goat-anti-mouse-Ig(total)-alkaline phosphatase diluted 500-2000 times in PBS containing 0.05% Tween 20, and 1% FCS, was added (50 µl/well) and the plates were incubated for 60 min at room temperature. After four washings with PBS containing 0.05% Tween 20, a solution of 4-methylumbelliferyl phosphate (0.2 mM in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$; 50 µl) was added as a substrate for alkaline phosphatase and the mixture was incubated at 37 °C for 30 min.
For the screening of supernatants in a direct ELISA against sulfur mustard adducts to keratin the same procedure was applied, with the following modifications. The plates were coated with keratin isolated from human callus treated with sulfur mustard (0 or 50 µM), dissolved in PBS to a final concentration of 5 µg/ml. After washing with PBS, the plates were incubated

with PBS containing 2% FCS, followed by washing with PBS containing 0.05% Tween 20 (three times). Hybridoma supernatants were screened in a 1:5 dilution. Further processing was the same as described above for plates coated with globin.

III.3.8 Immunoslotblot procedure for the detection of sulfur mustard adducts to keratin

Essentially, the procedure developed for the detection of sulfur mustard adducts to DNA (*12*) was applied. Briefly, a. solution of keratin (200 µl, 2.5 µg/ml PBS) isolated from a human callus sample exposed to sulfur mustard was spotted on a nitrocellulose filter. All samples were blotted in triplicate on the same filter. After blotting, the slots were rinsed with PBS. The filters were dried on air and the keratin was immobilized by UV-crosslinking (50 mJ/cm^2). Next, the filter was incubated with blocking solution (about 50 ml, 5% milkpowder in PBS + 0.1% Tween 20) at room temperature for 30 min and washed three times with PBS + 0.1% Tween 20. Next, the filter was incubated with 1st antibody (1H10, directed against sulfur mustard adducts to human keratin) diluted 500-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, overnight at 4 °C under continuous shaking, washed 4 times with PBS + 0.1% Tween 20, the last three times for at least 15 min each. Next, the filter was incubated with 2nd antibody (directed against the 1st antibody) diluted 1000-fold in 20 ml solution containing 0.5% milkpowder in PBS + 0.1% Tween 20, for 2 h at room temperature under continuous shaking, washed 4 times with PBS + 0.1% Tween 20, the last three times for at least 15 min each.

Finally, a 1:100 mixture of solution B with solution A (of the Enhanced Chemiluminescence Blotting Detection System) was preincubated in a water bath for at least 30 min at 25 °C. Then, free (wash) solution was removed from the filter with filter paper and position A12 and H1 marked with ball point (not a felt pen!). The filter was placed in a closely fitting box. After addition of substrate solution (10 ml) incubation was performed for 1 min.

Next, the filter was wrapped in plastic (straight from the substrate solution) without air bubbles. Liquid was pressed out, the filter was transferred in plastic into a luminometer cassette and placed in the luminometer. Luminescence was measured according to the required program. For each sample the mean luminescence of triplicate samples was calculated.

IV RESULTS

IV.1 Further development of the mass spectrometric analysis of the tripeptide (S-HETE)-Cys-Pro-Phe, resulting from pronase digestion of albumin alkylated by sulfur mustard

IV.1.1 Introduction

Results obtained within the framework of our previous grant DAMD17-97-2-7002 showed that pronase digestion of albumin alkylated by sulfur mustard resulted in the formation of the tripeptide (S-HETE)-Cys-Pro-Phe, which could be conveniently isolated and determined in a rather sensitive way by micro-LC/electrospray tandem MS with multiple reaction monitoring at an absolute detection limit of 4 pg (*11*). Using only 3 mg of albumin, we were able to detect *in vitro* exposure of human blood to 10 nM of sulfur mustard by applying this method. Presently, this is by far the most sensitive method for detection of exposure of human blood to sulfur mustard. Interestingly, we recently showed that this method can also be applied to demonstrate exposure (*in vitro* as well as *in vivo*) to a wide range of nitrogen mustard derivatives (*14*).

IV.1.2 Shortening of the procedure for albumin isolation by precipitation

The procedure for isolation of albumin from human blood samples, as routinely used in our laboratory, consists of overnight incubation of the plasma sample with $CaCl_2$ in order to precipitate the immunoglobulins, followed by a number of other washings and precipitation steps (*20*). This procedure could be substantially shortened by performing the incubation with $CaCl_2$ for only 1 h, instead of overnight. Identical levels of (S-HETE)Cys-Pro-Phe were found after pronase digestion, in comparison to albumin samples which had been isolated according to the original procedure.

IV.1.3 Isolation of albumin from plasma by affinity chromatography

It is clear that the isolation of albumin by means of precipitation is rather time-consuming, which limits the utility of the method. Moreover, the current isolation method precludes automation of the methodology. A literature research revealed that albumin can be removed from serum by affinity chromatography, which will facilitate the analysis of less abundant serum proteins (*21*). We reasoned that this procedure might be suitable for *isolation* of albumin from serum. The affinity material, having Cibacron Blue F3G-A as ligand, is commercially available in small columns. The affinity is based on specific interactions of the column material with amino acid residues in albumin.
In a representative experiment, 1 ml of serum or plasma (containing a theoretical amount of 40 mg of albumin) was applied to a 1 ml HiTrap Blue column. Plasma constituents with no affinity to the column material rapidly elute from the column, as evidenced by the UV pattern. Albumin is eluted from the column by applying a high salt buffer. This procedure takes 10 min. Subsequently, the fraction containing albumin (checked by UV) is desalted by means of gel filtration on a PD 10 column, which takes an additional 10 min. LC tandem MS analysis of pronase digests of the albumin fraction thus obtained gave similar results as found earlier by using the old procedure.

IV.1.4 Upscaling of pronase digestion of albumin

In previous experiments only 3 mg of albumin was digested with pronase, which enabled the detection of an exposure level of 10 nM. We reasoned that the use of larger amounts of albumin, in combination with purification of the pronase digest by means of Sep-Pak C18 cartridges, might lead to significantly lower observable exposure levels. Indeed, when 20 mg

samples of albumin were digested with pronase, followed by Sep-Pak clean-up of the digests, the lowest detectable exposure level could be improved by one order of magnitude (*i.e.*, 1 nM) (see Figure 2). However, the reproducibility of this procedure is not yet at the same level, compared with the 3 mg procedure. The micro-LC columns severely suffered from clogging, due to the relatively large amounts of material introduced. In future experiments more attention will be paid to further clean-up of the digests prior to the actual LC/electrospray tandem MS analysis on the triple-quad instrument.

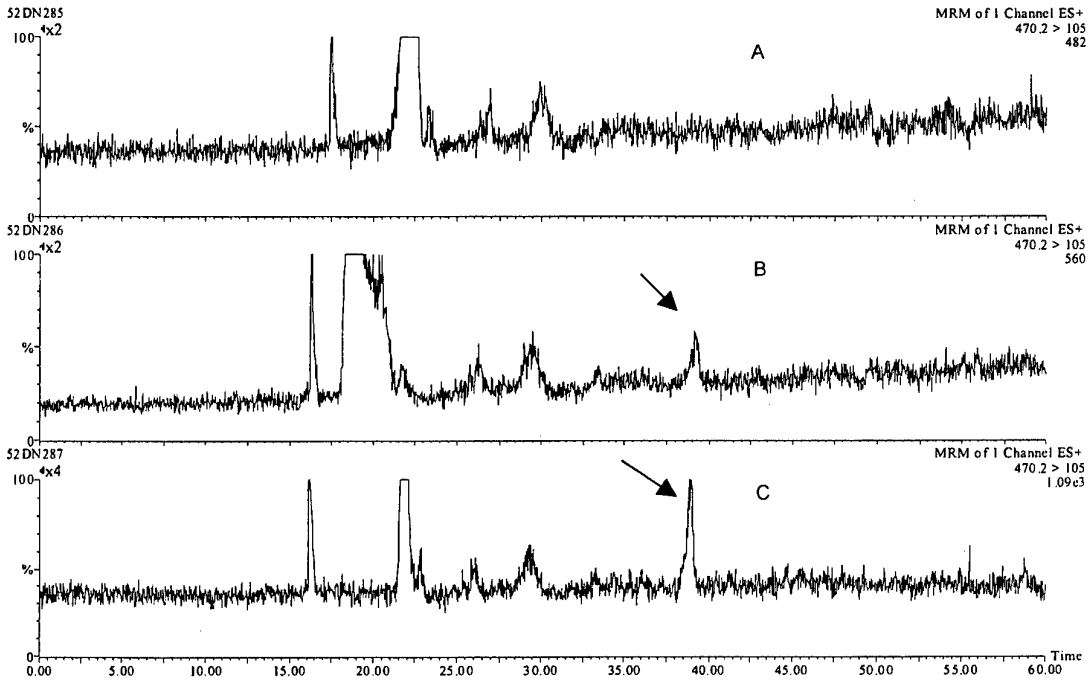


Figure 2. Trace level LC/electrospray tandem MS analysis of (S-HETE)Cys-Pro-Phe in pronase digest of albumin (20 mg) after purification on Sep-Pak C18, measuring the transition *m/z* 470 (MH^+) ---> 105. Albumin was isolated from non-exposed blood (A) or from human blood that was exposed to 1 nM (B). Panel C represents the 1 nM digest after spiking with synthetic (S-HETE)Cys-Pro-Phe. The arrow indicates the peak for (S-HETE)Cys-Pro-Phe.

IV.1.5 Use of albumin alkylated by d_8-sulfur mustard as an internal standard

The use of an internal standard, *i.e.*, albumin isolated from human blood exposed to d_8-sulfur mustard, has been worked out. This will enable quantitative analyses of unknown samples. With this procedure a stock solution in 4 M urea of powdered albumin, isolated from blood which had been exposed to 0.1 mM d_8-sulfur mustard is added to an "unknown" albumin sample, prior to pronase digestion. In this way, the internal standard can be added to the actual sample more accurately than by weighing. The use of d_8-sulfur mustard-alkylated albumin rather than the synthetically accessible d_8-tripeptide has the advantage that it provides evidence whether pronase digestion has indeed occurred. The tripeptide obtained after digestion of albumin, isolated from human blood that had been exposed to d_8-sulfur mustard, had similar mass spectrometric properties. Satisfactory calibration curves were obtained.

We also found that plasma, isolated from blood exposed to a well-defined amount of d_8-sulfur mustard, could be used as an internal standard in the assay which uses the affinity

chromatography procedure for isolation of albumin. This is more convenient than using a solution of powdered albumin (see Figure 3 for dose-response curve).

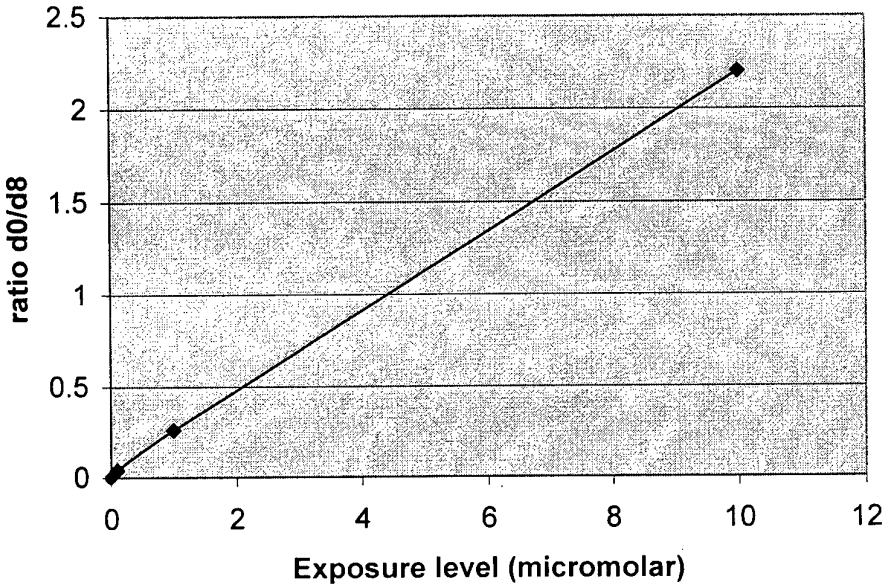


Figure 3. Dose – response curve for LC – tandem MS analysis after pronase digestion of albumin isolated by affinity chromatography from human blood that was exposed to various concentrations of sulfur mustard, in the presence of a fixed amount of internal standard which had been isolated from blood which had been exposed to d_8-sulfur mustard.

IV.1.6 Sector ion trap MS and Fourier transform – ion cyclotron resonance MS of (S-HETE)Cys-Pro-Phe

A number of analyses of pronase digests were run a sector ion trap instrument (BE ion Trap MAT900) at Thermoquest (Bremen, Germany). Unfortunately, the instrument used proved to be less sensitive than our Q-TOF instrument. The main reasons for this was the sample matrix and the deviating fragmentation pattern (less intense fragment *m/z* 105). An exposure level of human blood of 100 μM could still be detected.

Further analyses were run on an FT-ICR instrument at the Environmental Molecular Sciences Laboratory (EMSL; Richland, Washington). Pronase digests of human serum albumin were prepared. Since we were not allowed to store the samples on dry ice during the flight to USA, we tested the stability of the digests. Digests were stored in 4 ml glass vials at room temperature and were analyzed each day. The amount of alkylated tripeptide did not decrease during storage. However, when the same samples were stored in eppendorf tubes, the presence of alkylated tripeptide could not longer be demonstrated after three days! After optimization of the instrument (11.5 Tesla FT instrument), the alkylated tripeptide could be determined with a detection level of approximately 200 ng/ml (2 ng absolute), which is a factor of 10 less sensitive than our Q-TOF instrument. In a pronase digest the adduct could not be determined. Altogether, it seems that the FT-ICR technique is not a good technique for measurement of trace amounts of low-molecular analytes in complex biological matrices.

IV.1.7 Derivatization of (S-HETE)Cys-Pro-Phe for LC tandem MS analysis

It has recently been reported that mass spectrometric detection of fluorinated amino acid derivatives under Electron Capture Atmospheric Pressure Chemical Ionization (EC APCI) conditions results in far better detection limits, when compared to normal electrospray MS (*19*). We were anxious to find out whether this technique could also be applied to analysis of (S-HETE)Cys-Pro-Phe. Thus, derivatization of (S-HETE)Cys-Pro-Phe with pentafluorobenzyl bromide (PFB-Br) in the presence of KOH in acetonitril afforded predominantly the di-PFB derivative of the tripeptide, with the PFB groups on the amino group and on the carboxylic acid group (Figure 4). Preliminary mass spectrometric analyses indicated that at least for the synthetic reference compound, the fluorinated derivative could be determined slightly more sensitive than the underivatized compound. Care has to be taken however: only standard solutions of the purified derivative have been analyzed in this stage. Derivatization yields and the effect of a matrix, *i.e.*, a mixture of other derivatized amino acids, have not been taken in consideration yet.

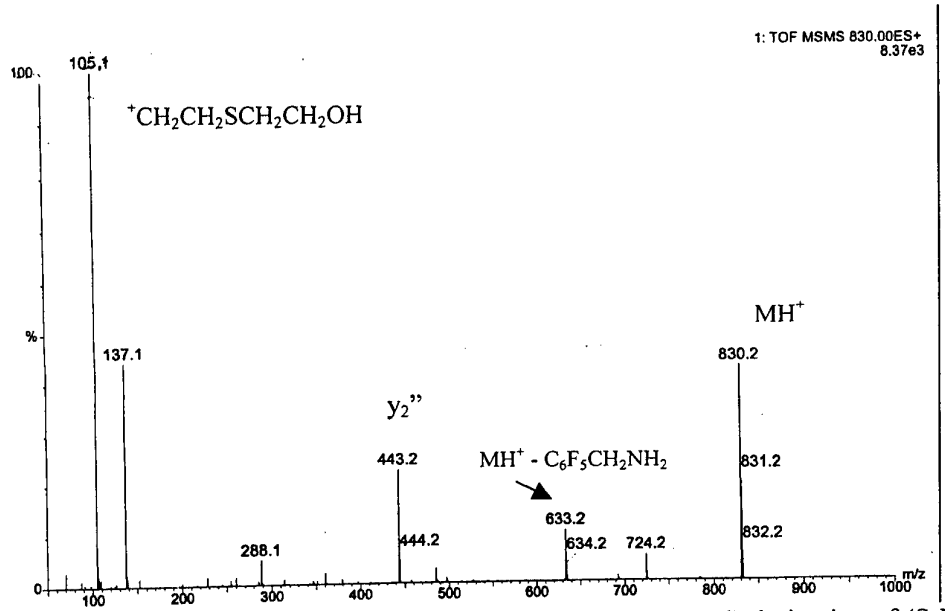


Figure 4. Tandem MS spectrum of bis(pentafluorobenzyl) derivative of (S-HETE)Cys-Pro-Phe.

IV.1.8 Persistence of albumin adducts

In order to obtain information about the persistence of the albumin – sulfur mustard adduct, laboratory animals will be exposed to the agent. We prefer to use the rat for these studies because the amino acid sequence of rat albumin has been published. Furthermore, it has been reported that the modified tripeptide Cys-Pro-Tyr results from rat albumin modified at Cys-34 by the food-borne carcinogens IQ (*22*) and PhIP (*23*), after pronase treatment. Preliminary experiments, in which rat blood had been exposed *in vitro* to sulfur mustard, showed that the major product was (S-HETE)-Cys-Pro-Tyr (see Figure 5 for tandem MS spectrum), and that the level of alkylation of the free cysteine residue in rat albumin was approximately 5%. Electrospray tandem mass spectrometric analyses could be performed in an analogous way, *i.e.*, by selecting the charged molecular ion in the first MS and measuring the highly selective 105 fragment in the second mass spectrometer. Consequently, the rat will be chosen as laboratory animal for studying the persistence of the albumin adduct.

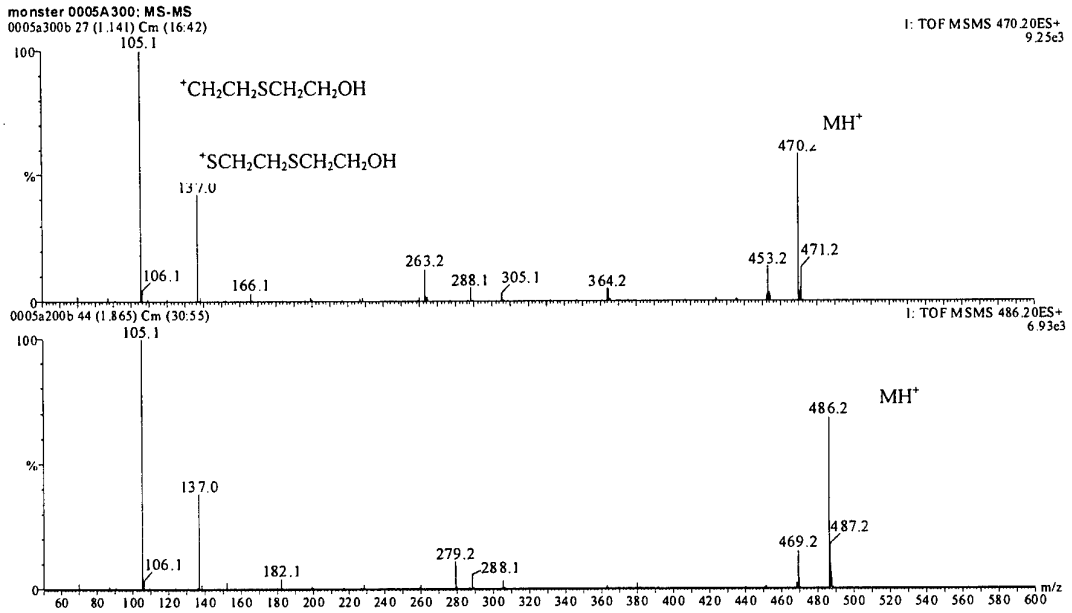


Figure 5. Tandem MS spectra of (S-HETE)Cys-Pro-Phe (upper panel) and (S-HETE)Cys-Pro-Tyr (lower panel).

In the mean time, we have performed some experiments with plasma of marmosets that had been exposed to sulfur mustard. These plasma samples had been isolated from blood samples taken during experiments for determination of the persistence of hemoglobin adducts, within the context of our previous cooperative agreement DAMD17-97-2-7002, and had been stored for 2 years at –70 °C. Albumin was isolated from these samples using the precipitation procedure. Unfortunately, the amino acid sequence of marmoset albumin is not known. Nevertheless, we digested small amounts (3 mg) of the albumin samples and analyzed the digests for the presence of the (S-HETE)Cys-Pro-Phe. The first three samples (1 h, 1 day, and 7 days after administration of 4.1 mg/kg sulfur mustard, iv) were digested and analyzed without the use of an internal standard. The presence of (S-HETE)Cys-Pro-Phe could be demonstrated, which indicates that marmoset albumin contains a free cysteine function near Pro-Phe, which is prone to alkylation by sulfur mustard. The levels after 1 h and 1 day were comparable, while the sample taken after 7 days was approximately 1.6 less intense. The other samples (*i.e.*, taken after 14, 21, 28 and 56 days) were digested and analyzed in the presence of an internal standard (albumin from human blood that was exposed to d_8-sulfur mustard). The estimated exposure levels were 1.49, 0.76 and 0.37 µM (compared to the internal standard) for the 14, 21, and 28 days samples, respectively (see Figure 6). In the sample which had been taken after 56 days, the observed peak was below the detection limit. It must be stressed, however, that the digests had not been pre-purified by means of Sep-Pak C18 and that the analyses were run on a Q-TOF instrument instead of triple-quad instrument, which has higher sensitivity. The plasma samples from the other experiment with the marmoset will be analyzed on a triple-quad instrument, which will probably give a similar curve as the one obtained for the N-terminal valine adduct (see Final Report of Cooperative Agreement DAMD17-97-2-7002).

When we assume that alkylation does not influence the life time of the protein, we can derive that the half-life time of marmoset albumin is 7 days (for humans: 20-25 days).

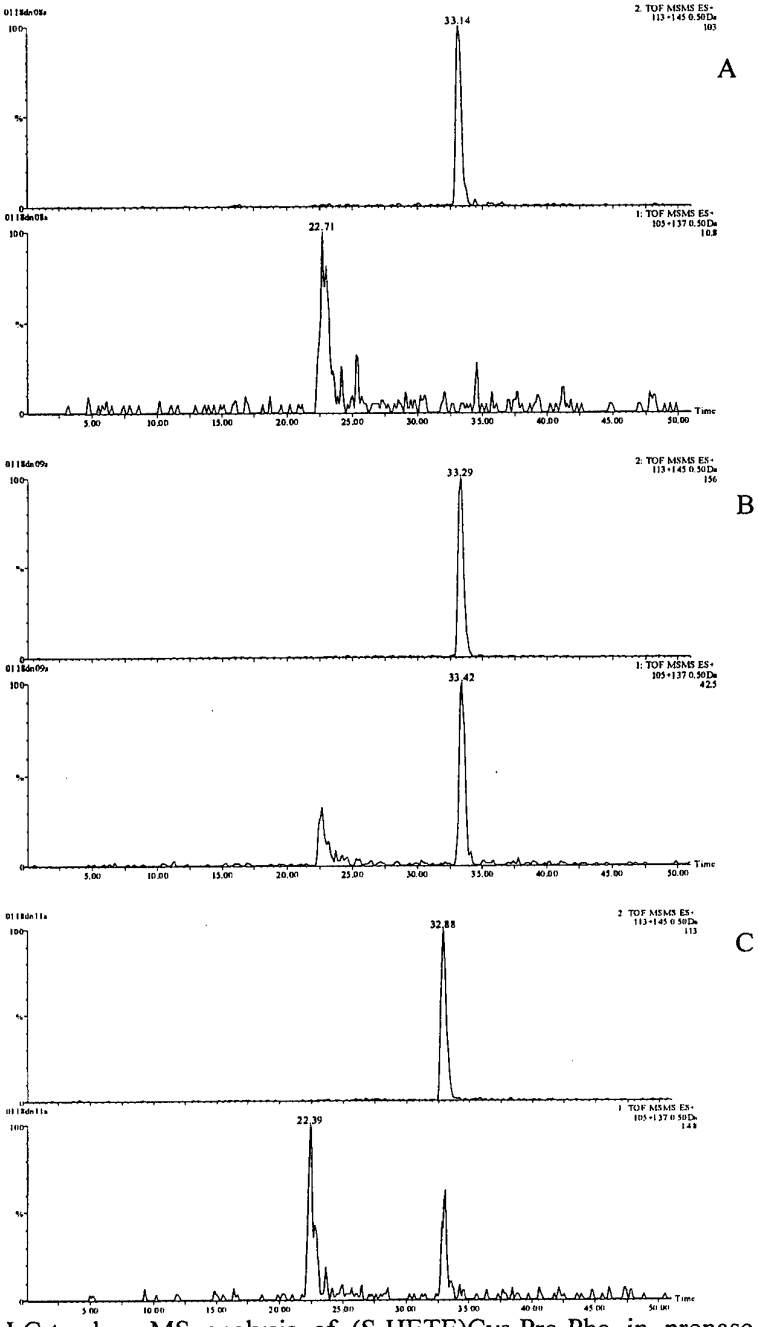


Figure 6. LC-tandem MS analysis of (S-HETE)Cys-Pro-Phe in pronase digests of albumin isolated from plasma taken from a marmoset which had been exposed (i.v.) to sulfur mustard (4.1 mg/kg). The sample was analyzed in the presence of albumin, isolated from human blood which had been exposed to d_8-sulfur mustard (corresponding with a level of 5 μM d_8-sulfur mustard). Panel A: internal standard + blank marmoset albumin sample. Panel B: internal standard + marmoset albumin from blood sample taken 14 days after intoxication. Panel C: internal standard + marmoset albumin from blood sample taken 28 days after intoxication. In each panel (A-C), the upper trace represents (S-d_8-HETE)Cys-Pro-Phe (resulting from digestion of d_8-sulfur mustard-alkylated albumin), while the lower trace represents (S-HETE)Cys-Pro-Phe (resulting from digestion of albumin from the marmoset).

IV.2 Further development of methods for sensitive analysis of N1/N3-(2-hydroxyethylthioethyl)histidine

IV.2.1 Introduction

In hemoglobin and albumin from human blood exposed to sulfur mustard, 34% and 28% of total adduct level could be ascribed to N1/N3-HETE-histidine, respectively (*9*). In view of this abundance and the stability of the adduct under acidic conditions, which enables its quantitative release from proteins, we investigated the mass spectrometric analysis of this adduct for which no sensitive method is currently available. The only published method is based on LC/electrospray tandem MS analysis of the 9-fluorenylmethoxycarbonyl (Fmoc) derivative of N1/N3-HETE-histidine, which allowed the determination of an exposure level of human blood *in vitro* of 10 μM sulfur mustard from the adducts formed in hemoglobin (*9*).

IV.2.2 Development of a work-up procedure for isolation of N1/N3-HETE-histidine

We developed a work-up procedure for isolation of the histidine-sulfur mustard adducts from amino acid mixtures resulting from acidic hydrolysis of hemoglobin or albumin. This procedure consists of an ion chromatography step by using Dowex 50 WX8 (Na^+ form). In this way, uncharged amino acids were eluted with aqueous acetic acid. After elution with water, elution with diluted NH_4OH afforded purified histidine adducts, which were further derivatized with Fmoc-Cl. Further clean-up could be achieved by elution on a Seppak C18 cartridge (see Figure 7).

IV.2.3 Hydrazinolysis of globin and analysis of HETE-histidine hydrazine derivatives

As an alternative to acidic hydrolysis of globin, it was attempted to degrade globin by means of hydrazinolysis, *i.e.*, by treatment with hydrazine, as was reported by Helleberg et al. (*24*) We were anxious to find out whether the resulting HETE-histidine hydrazine derivative has more favorable properties for LC-MS or GC-MS analysis than the native adduct. Treatment of N1-HETE-histidine methyl ester with hydrazine monohydrate in isopropanol, resulting in the desired HETE-histidine-N_2H_4 derivative. Treatment of globin with hydrazine monohydrate resulted in the formation of the same HETE-histidine-N_2H_4 derivative, as well as HETE-histidine-OH derivative, as evidenced by LC-tandem MS analysis. Pure hydrazine should probably give only the hydrazide derivative. The hydrazide derivative proved to be highly unstable, even towards solvents as THF, and could not be further derivatized for GC-MS analysis. We decided to skip further work on hydrazinolysis and analysis of HETE-histidine hydrazide derivatives.

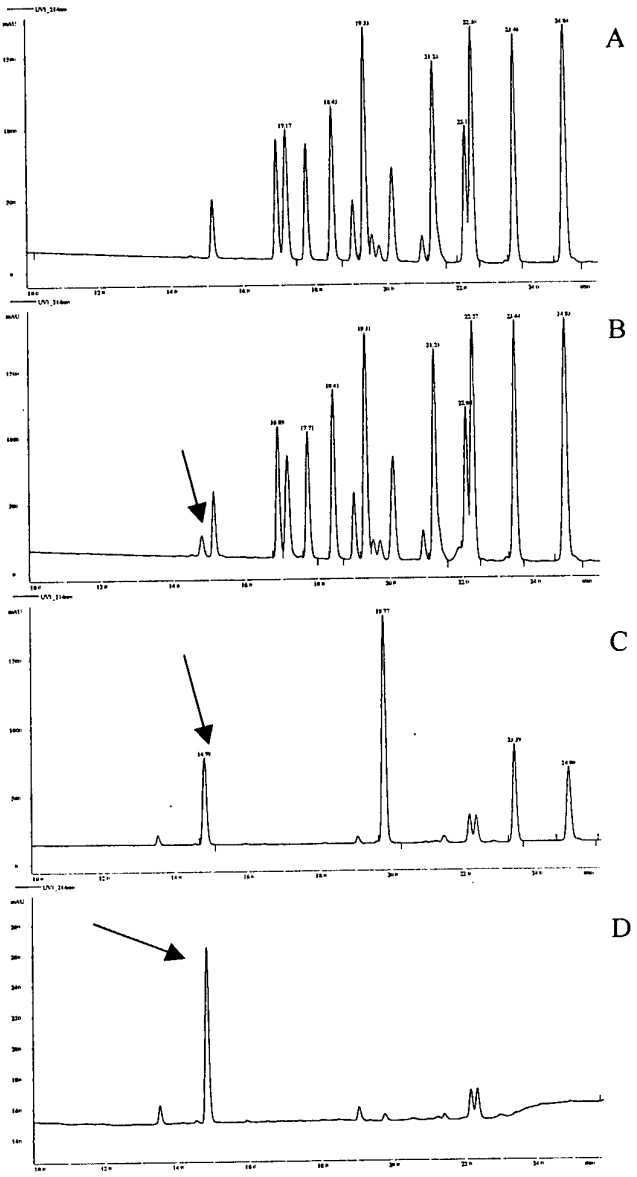


Figure 7. HPLC analysis of acidic digests of human globin, spiked with synthetic N1/N3-HETE-histidine, after derivatization with Fmoc-Cl. (A) non-spiked acidic globin hydrolysate, (B) globin acidic hydrolysate spiked with N1/N3-HETE-histidine, (C) globin acidic hydrolysate spiked with N1/N3-HETE-histidine after pre-purification by cation-exchange chromatography, and (D) after purification of the digest of panel C on Sep-Pak C18. The arrow indicates the peak for the Fmoc-N1/N3-HETE-histidine derivatives.

IV.2.4 Derivatization of N1/N3-HETE-histidine for GC-MS analysis

From previous research (*9*) it appeared that GC-MS based procedures for analysis of the histidine adducts were troublesome, mainly due to the high polarity or thermal instability of the derivatives. It was found that reaction of the methyl ester of the histidine adducts with trifluoroacetic anhydride afforded a derivative which could be analyzed in a rather sensitive

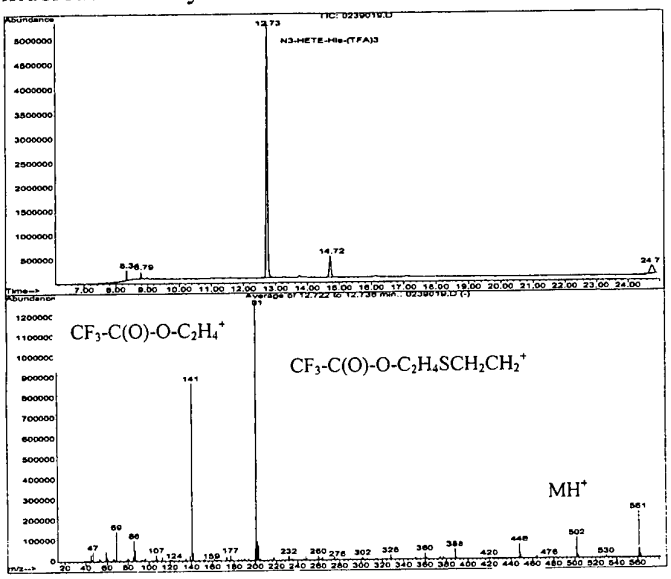


Figure 8. GC-EI-MS analysis of tris(trifluoroacetyl)-N3-(HETE)-histidine.

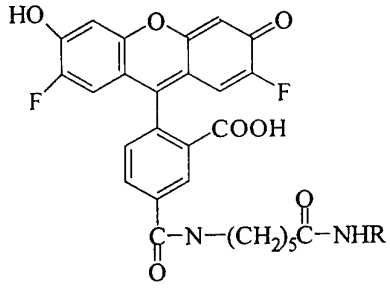
way by GC-MS (detection limit: 3 ng/ml; 3 pg absolute); see Figure 8. Reaction of N3-HETE-histidine methyl ester with heptafluorobutyric anhydride gave rise to the formation of a bis(heptafluorobutyryl)derivative. However, reaction of N1-HETE-histidine methyl ester with heptafluorobutyric anhydride did not proceed. Reaction with pentafluoropropionic anhydride gave the corresponding bis(pentafluoropropionyl)derivative.
Derivatization with trifluoroacetic acid anhydride was selected for future experiments.

IV.2.5 Derivatization of N1/N3-HETE-histidine for LC-tandem MS analysis

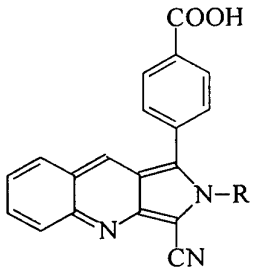
In our previous cooperative agreements we found that derivatization of N1/N3-histidine with Fmoc-Cl afforded the corresponding Fmoc derivative that could be analyzed rather sensitively by means of LC-tandem MS (*9*). Recently, it has been reported (*19*) that mass spectrometric detection of fluorinated amino acid derivatives under EC APCI conditions (vide supra) results in far better detection limits, when compared to normal electrospray MS. Derivatization of N1/N3-HETE-histidine with pentafluorobenzyl bromide in DMF, under the agency of triethylamine, afforded the corresponding tris-pentafluorobenzyl (PFB) derivatives. Unfortunately, we were not able to analyze these derivative with GC-MS. ^{1}H-NMR analysis of the derivative showed that the imidazole function had been modified with a PFB group. This might explain the failure of GC-MS analysis: such a derivative has a permanent positive charge. Fortunately, this derivative exhibited favorable properties for LC/electrospray tandem MS analysis. It has a long retention time, compared to other derivatives, which might enable Sep-Pak C18 clean-up prior to the actual MS analysis. In future experiments it will be investigated whether the tris(PFB) derivatives can also be analyzed under EC APCI conditions.

IV.2.6 Derivatization of N1/N3-HETE-histidine for HPLC with laser-induced fluorescence detection

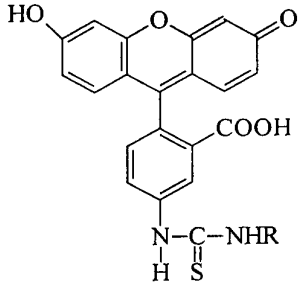
Laser-induced fluorescence has been used for detection of yoctomole amounts of amino acid derivatives, whereas detection at single molecule level has been shown, albeit under extremely controlled conditions (*25*). This technique, however, is less selective than mass spectrometry. Various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared. Three derivatization reagents were used for labelling of N1/N3-(2-hydroxyethylthioethyl)histidine (see Figure 9). Fluorescein-5-isothiocyanate (FITC 'isomer I') was selected because it is one of the most widely used fluorescent dyes. Isothiocyanates form reasonably stable thioureas upon reaction with amines. A drawback is its relatively low reactivity. FITC derivatized amine groups have an excitation maximum at 492 nm and an emission maximum at 520 nm. 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) was selected because of the advantage that the unreacted dye is non-fluorescent. The reaction with primary amines involves an intramolecular ring closure, in the presence of cyanide ions. CBQCA derivatized amine groups have an excitation maximum at 468 nm and an emission maximum at 560 nm.
Oregon Green 488-X, succinimidyl ester, 6-isomer is a fluorescein derivative. Conjugates of Oregon Green 488 dyes have several advantages compared to conjugates of fluorescein. These include greater photostability, higher fluorescence and less quenching. In this particular case the succinimidyl ester was used because it is well-known that succinimidyl esters are more reactive than isothiocyanates. For all abovementioned fluorescent dyes the desired conjugates with N1- and N3-(2-hydroxyethylthioethyl)histidine could be prepared in good yields.
The derivatives were isolated by semi-preparative FPLC, using a reversed-phase column, and characterized with electrospray MS. Surprisingly, the derivatives of FITC slowly rearranged into compounds which are probably the corresponding thiohydantoins (based on mass spectrometry). The derivatives were stored as dry solids and proved stable for several months. With the fluorescent derivatives in hand, the detection limits will be determined for the derivatives and the derivatization of amino acid mixtures (containing the adducts) will be studied in more detail.



FITC derivative

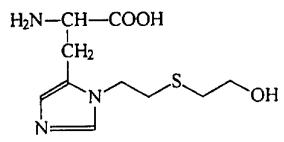


CBQCA derivative

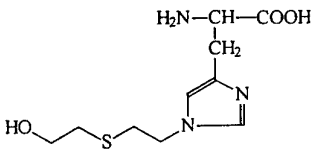


Oregon Green derivative

R = N1/N3-(2-hydroxyethylthioethyl)histidine



N1-isomer



N3-isomer

Figure 9. Fluorescent derivatives of N1/N3-(2-hydroxyethylthioethyl)histidine.

IV.3 Optimization of immunoassay for screening of available antibodies against sulfur mustard adducts to proteins

IV.3.1 Introduction

The following steps were taken in our general approach to the development of an immunochemical assay for the detection of sulfur mustard adducts with proteins. It has been attempted to further improve the sensitivity and reproducibility of the ELISA for the screening of monoclonal antibodies against sulfur mustard adducts to hemoglobin, albumin and keratin. Next, some of the most promising clones were further characterized and applied in the development of the generally more sensitive immunoslotblot assay.

IV.3.2. Screening of monoclonal antibodies directed against sulfur mustard adducts to hemoglobin and globin in a direct ELISA

An ELISA assay was set up for the screening of antibodies against sulfur mustard adducts to hemoglobin. The antibodies were supplied as supernatants of clones obtained from fusions after immunization of mice with peptide haptens containing a histidine-sulfur mustard adduct derived from human globin or which have been adducted with sulfur mustard to a cysteine residue (*12*). The following procedure was applied:

1. Coating of plates with hemoglobin (50 µl of 5 µg/ml, isolated from human blood exposed to 0 and 50 µM sulfur mustard) in PBS for 1 h at room temperature,
2. washing with PBS (3 times),
3. blocking of free places with 2% FCS in PBS (30 min at room temperature),
4. washing with PBS + 0.05% Tween (3 times),
5. adsorption of 1st ab (in duplicates): supernatants of clones obtained from sulfur mustard-his-peptides fusions, and of 3H6, obtained from a mouse immunized with an alkylated peptide, *i.e.*, N-acetyl-S-HETE-cys_{93} through leu_{106}-lys of the β-chain of hemoglobin (*4*), were applied in a 1:5 dilution, 60 min at room temperature,
6. washing with PBS + 0.05% Tween (3 times),
7. adsorption of 2nd ab: 1/1000 diluted GAM-alkaline phosphatase in PBS+0.05% ween+1% FCS, 55 min at room temperature,
8. washing with PBS + 0.05% Tween (3 times), and with DEA (1 time),
9. incubation with MUP in reaction buffer, pH 9.8, 45 min at 37 °C,
10. measurement of fluorescence with microtiter plate reader.

Supernatant of clone 3H6 did not show any specificity for alkylated hemoglobin. Also other supernatants from clones from immunizations with an adducted his-peptide derived from hemoglobin, did not show any specificity for alkylated hemoglobin. Because there was some doubt about the alkylated hemoglobin used for binding to the wall of the well on the microtiter plate we isolated new hemoglobin and globin from blood exposed to sulfur mustard. Also with these antigens no positive result was obtained.

Only one clone, 183 3D5-2E11, showed some specificity for alkylated hemoglobin and alkylated globin.
When coated with alkylated hemoglobin at a higher concentration (10 µg /ml PBS instead of 5 µg/ml), still no specificity was observed for clone 3H6 and clones derived from immunizations with the his-peptides 183 and 190.

Another clone, Val-1H2 (directed against the sulfur mustard adduct to the N-terminal valine of the α-chain of globin) showed some specificity against alkylated globin on plates pre-coated with glutaraldehyde.

These puzzling data stressed the need to study the different steps in the immuno assay in more detail. Parameters varied were the following:

A Sub-culturing of clone Val-1H2, to select monoclonals with freshly prepared supernatants.
B Concentration of hemoglobin or globin used for coating: 5, 10 or 20 µg/ml.
C Coating with hemoglobin in water or PBS.
D Coating with hemoglobin, old and fresh.
E Coating with globin, old and fresh.
F Coating with hemoglobin and globin over a larger exposure range (0-1 mM sulfur mustard).
G Coating with trypsinized albumin and globin.
H Pre-coating with glutaraldehyde (5%) or poly-l-lysine (100 µg/ml) (60 min at room temperature).
I Pre-coating with glutaraldehyde (0.1, 0.2 and 1%) or poly-l-lysine (10 or 100 µg/ml) (o/n at 4 °C), and coating with globin (1, 5, 10 or 20 µg/ml, 1 h at room temperature).
J Application of BioStab immunoassay stabilizer (after pre-coating with 0.2% glutaraldehyde and coating with globin, 10 µg/ml); no wash after addition of BioStab.
K Coating overnight at 4°C, at 37 °C dry, at 37 °C wet or for 1 h at room temperature with continuous vibration.
L Coating in bicarbonate, pH 9.6, instead of PBS.
M Blocking of free places with water + 5% FCS or PBS + 2% FCS.
N Variation of time and temperature of treatment with 1st ab and 2nd ab.

The following results were obtained:

Sub-cloning of clone Val-1H2

Several monoclonals were selected after subcloning of clone Val-1H2. Two of these, IIIE8 and IIIG10, were selected and their supernatants used for further optimization of the immuno assay.

Variation of coating conditions

- Although originally positive results were obtained when applying pre-coating with glutaraldehyde, in later experiments this result could not be reproduced. Even, pre-coating with glutaraldehyde, followed by coating with globin, resulted in enhancement of a-specific binding of antibody.
- Coating with hemoglobin (5 or 10 µg/ml) instead of globin resulted in extreme high (a-specific) fluorescence, even with 1:500 diluted supernatant (to the same extent as with the combination glutaraldehyde and globin).
- Pre-coating with poly-l-lysine resulted in high background fluorescence.
- Application of BioStab resulted only in a marginal improvement.
- Coating in bicarbonate instead of PBS appeared to be not an improvement.
- The concentration of globin during coating appeared to be important: 10 µg/ml gave good results both with coating for 1 h or overnight (wet), whereas with 5 µg/ml in combination with coating for 1 h at room temperature a reverse effect was observed and with coating overnight (wet) strong non-specific adsorption of antibody occurred.
- The blocking of free places with FCS appeared to be efficient as could be concluded from the low background fluorescence when only 2nd ab was added to the coated plates (without pre-coating and with coating in PBS).

Variation of time and temperature of treatment with 1st ab and 2nd ab

The best results were obtained when adsorption of both 1st ab and 2nd ab occurred for a short period (30 min) at room temperature. Upon application of the conditions which facilitate adsorption (optimal binding temperature, 37 °C, and longer incubation periods, 1h), then a-specific binding of 1st ab to globin) was preferentially increased. Enhancement of globin concentration (to 12.5 µg/ml) resulted in a lower specific fluorescence, but a-specific binding was still more decreased. Under these conditions, even at a 1:2000 dilution of 2nd ab a distinct difference in fluorescence was observed between non-alkylated globin and globin alkylated with 100 µM sulfur mustard.

These data suggest that the immuno assay for screening of monoclonals producing ab's against sulfur mustard adducts to globin can still further be improved by suppressing the a-specific binding of 1st ab to globin, for example by carrying out the 1st ab adsorption at a higher concentration of Tween 20. When this results in a more sensitive and more reproducible assay than, after preparing fresh supernatant of each clone, all other available monoclonal clones can be screened for efficiency of ab- binding.

IV.3.3 Screening of monoclonal antibodies directed against sulfur mustard adducts to albumin in a direct ELISA

During the past period no supernatants of clones have been screened obtained from immunizations with alkylated peptides of human serum albumin

IV.3.4 Screening of monoclonal antibodies directed against sulfur mustard adducts to keratin in a direct ELISA

An ELISA assay was set up for the screening of antibodies against sulfur mustard adducts to keratin. More or less the same procedure was applied as originally applied for the screening of anti-sulfur mustard-globin clones, described in section IV.2.1., except that coating of plates was carried out with keratin (50 µl of 5 µg/ml, isolated from human callus exposed to 0 and 50 µM sulfur mustard) in PBS for 1 h at room temperature.

Several clones could be selected with a high specificity for keratin exposed to sulfur mustard. A number of these are presented in Table 1.

Other supernatants appeared to have lost their activity. For that reason 28 clones, all originating from the same clone 1.3-C2 from the mouse immunized with peptide 1 + 2, were cultured again to produce fresh supernatant. Although there was some differentiation in growing rate of the cell cultures, all supernatants appeared to be active, indicating that all clones were still able to produce antibodies specific for sulfur mustard adducts to keratin.

IV.4 Cross reactivity of antibodies for sulfur mustard adducts with hemoglobin, albumin and keratin.

Clone 3H6, which had been derived from a fusion with an adducted cysteine of a peptide derived from hemoglobin, showed some specificity for both alkylated keratin (Table 1).

The same clone, and some clones derived from fusions with an adducted his-peptide derived from hemoglobin did not show any specificity for alkylated human serum albumin. However, when applied on microtiter plates coated with alkylated keratin some clones derived from fusions with an adducted his-peptide derived from hemoglobin, showed some binding (Table 2; see also section IV.2.1). This suggests that the specificity depends in some cases mainly on the presence of the adduct and not on the amino acid to which the adduct is bound.

Table 1. Antibody specificities of clones obtained from a fusion after immunization with haptenes of partial sequences of keratin containing glutamine (1,2) or asparagin (3)-sulfur mustard adduct. Supernatants of cultures in a 1:5 dilution were assayed in a direct ELISA on keratin from human callus treated with 0 μM or 50 μM sulfur mustard. Fluorescence (in arbitrary units) is presented as a measure for antibody binding.

clone	peptides used for immunization	Antibody response against keratin exposed to sulfur mustard solution of .	
		0 μM	50 μM
2.3D7	3	124	537
3.2G8	1+2+3	294	1165
1.1A8		2969	4013
1.2B6	1+2	275	1445
2.3D9	3	337	2709
2.2B5	3	494	3380
1.3C2-1D9	1+2	810	3423
1.3C2-1B4	1+2	641	3766
1H10	1+2	975	3791
1H10(2)	1+2	618	3745
3H6		586	1268

Table 2. Antibody specificities of clones obtained from a fusion after immunization with haptenes of partial sequences of hemoglobin containing histidine-sulfur mustard adduct. Supernatants of cultures in a 1:5 dilution were assayed in a direct ELISA on keratin from human callus treated with 0 μM or 50 μM sulfur mustard. Fluorescence (in arbitrary units) is presented as a measure for antibody binding.

clone	Peptide* used for immunization	Antibody response against keratin exposed to sulfur mustard solution of .	
		0 μM	50 μM
D5-2E9	1	228	2628
4A3-1B3	3	238	2891
4A3-1C2	3	246	2668
4A3-2A10	3	299	2943
4A3-2B7	3	293	2996
4A3-2F4	3	327	2969
4A3-2F6	3	240	2498
4A3-1A7	3	160	1623
4A3-2G7	3	135	1578
4A3-2E8	3	397	3274

*These peptides (1: AFSDGLA(N1/N3-HETE)HLDNLK, corresponding with residues 70-82 of human β-globin; 3: L(N1/N3-HETE)HVDPENFRLLGNVK, corresponding with residues 96-109 of human β-globin) have been described in detail previously (*12*).

Some clones specific for alkylated keratin (see Table 1) showed also a weak affinity for alkylated human serum albumin. Unfortunately, this result could not be reproduced.

IV.5 Characterization of monoclonal antibodies directed against sulfur mustard adducts to proteins

IV.5.1 Antibodies against sulfur mustard adducts to keratin

Nine clones producing antibodies against sulfur mustard adducts to keratin were selected for further characterization and subcloning. As described previously, 32 monoclonal clones, all originating from clone 1.3C2, have been selected of which antibodies showed specificity against keratins isolated from human callus treated with 50 μM sulfur mustard (12).
Antibodies of one clone, 1H10, were characterized further. Cross-reactivity toward the 3 hapten peptides (GVVSTH(N-ω-HETE)QQVLRTKNK (1); GIQ(N-ω-HETE)QVTVNQSLLTPLNK (2) and GVM(Nω-HETE)NVHDGKVVSTHEK (3)) and the corresponding nascent peptides was assessed in a competitive ELISA (Table 3).

Table 3. Antibody specificity of clone 1H10 obtained from a mouse immunized with peptide 1+2

50% inhibition in competitive ELISA with		
	sulfur mustard-peptide (nmol/well)	Nascent-peptide (nmol/well)
Peptide 1	>20	>20
Peptide 2	1.4	6.5
Peptide 3	7.6	>20

With 1.4 nmol/well of peptide 2 50% inhibition was observed, whereas with the corresponding nascent peptide 6.5 nmol/well was required to achieve the same extent of inhibition. Peptide 3, which was not used for the immunization resulting in clone 1H10, also showed some cross-reactivity (50% inhibition with 7.6 nmol/well). All other peptides tested, including peptide 1 used for the immunization, did not show any cross-reactivity in the concentration range tested, *i.e.*, 50% inhibition is not achieved at amounts less than 20 nmol/well.

IV.6 Development of the immunoslotblot assay for the detection of sulfur mustard adducts to proteins

IV.6.1 Sulfur mustard adducts to hemoglobin

As described previously, several clones of which the antibodies recognized alkylated hemoglobin were obtained from mice immunized with an alkylated peptide, *i.e.*, N-acetyl-S-HETE-cys_{93} through leu_{106}-lys of the β-chain of hemoglobin (*4*). One of these, 3H6, was further characterized. These antibodies recognized sulfur mustard-modified hemoglobin in a dose-dependent way. It appeared that exposure of human hemoglobin to 50 μM sulfur mustard was detectable in a direct ELISA (*4*). However, the direct ELISA is usually not the most sensitive immunochemical assay. Therefore, we have attempted to apply these antibodies to an immunoslotblot assay for alkylated hemoglobin, but so far without lowering the minimum detectable concentration.

IV.6.2 Sulfur mustard adducts to human keratin

We applied the immunoslotblot assay, developed for the detection of N7-HETE-Gua in DNA, on sulfur mustard adducts with keratin using the antibodies 1H10 directed against these

adducts. Keratin was isolated from human callus exposed to sulfur mustard. In Figure 10 the data are summarized.

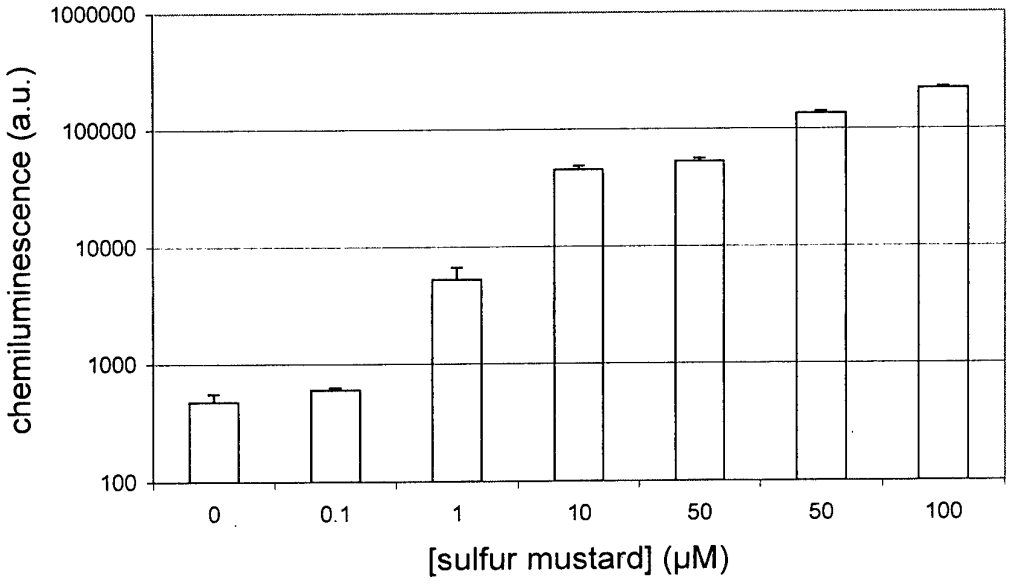


Figure 10. Immunoslotblot assay on keratin of sulfur mustard exposed human callus. Human callus was exposed to sulfur mustard at concentrations ranging from 0 to 100 μM sulfur mustard. Keratin was isolated and applied in 0.5 μg aliquots in the assay. Data are the mean of triplicates. The error bars represent the standard error of the mean.

V DISCUSSION

Development of a Standard Operating Procedure for determination of sulfur mustard protein adducts; albumin adducts

In this grant period we succeeded in significantly shortening the procedure for albumin isolation and subsequent pronase digestion. First, we found that the precipitation step with $CaCl_2$ can also be performed for only one hour, instead of overnight. In the end, *i.e.*, after pronase digestion and LC-tandem MS analysis, the same results were obtained. Furthermore, we found that a commercially available affinity column for albumin could be advantageously used in the tentative standard operating procedure. After the affinity chromatography procedure and rapid desalting of the albumin on a PD10 column, the purified albumin sample is recovered as a solution in aqueous NH_4HCO_3. The thus obtained solution can be used immediately for pronase digestion. In the next grant period, we plan to use columns with immobilized pronase. This might enable the construction of an automated system of (reactor) columns, coupled to a tandem mass spectrometer, in which an unprocessed plasma sample can be introduced. This would be highly convenient for use under field laboratory conditions, and more importantly, would speed up the actual diagnosis.

The use of an internal standard, *i.e.*, a plasma sample isolated from blood that had been exposed to d_8-sulfur mustard, has also been worked out in combination with the affinity chromatography procedure. In previous experiments we used a 4 M urea solution of powdered d_8-sulfur mustard alkylated albumin. However, we found that the precipitated protein had poor solubility in 4 M urea. Moreover, the presence of large amounts of urea has negative effects on the LC-tandem MS analyses of the pronase digest.

The minimal observable exposure level (in vitro) could be lowered a factor of 10, *i.e.*, 1 nM, by using larger amounts of albumin (20 mg instead of 3 mg). However, due to the large amounts of peptide material injected the micro-HPLC column was often overloaded and showed poor separation characteristics. In the next grant period this will be further optimized.

In preliminary in vitro experiments with [^{14}C]-labelled sulfur mustard we found that the free cysteine in rat albumin is also prone to alkylation by sulfur mustard. It was found by LC-tandem MS analysis that a tripeptide (S-HETE)Cys-Pro-Phe was formed after pronase digestion. The level of alkylation at the particular cysteine residue was approximately 5%. On the basis of these results we believe that the rat is a good animal for studying the persistence of the adduct.

Within the context of our previous cooperative agreement marmosets had been exposed to sulfur mustard and blood samples had been taken at regular time points for determination of the N-terminal valine adduct to sulfur mustard. It appeared that the N-terminal valine adduct was still detectable 94 days after the exposure had occurred. We had stored the plasma samples (we had only used the erythrocytes in our previous studies) in the freezer and we were anxious to find out whether we could determine the albumin adducts. Remarkably, the amino acid sequence of marmoset albumin is not known, so actually we did not really know whether there is any free cysteine residue present in this protein. After pronase digestion of a small amount of albumin from the exposed marmoset, the tripeptide (S-HETE)Cys-Pro-Phe could be analyzed. As should be expected, the particular albumin adduct was quite stable in the marmoset in vivo, probably as stable as the protein itself. The adduct could still be determined after at least 28 days after the exposure. It has to be remarked, however, that the analyses were run on a Q-TOF instrument, and not on a triple-quad instrument, which has generally more sensitivity. When it is assumed that the life-time of albumin is not affected by alkylation with sulfur mustard, the half-life of marmoset albumin is approximately 7 days. Only a few samples were analyzed in the presence of an internal standard. Further quantitative analyses of these samples will be performed in the next grant period.

Development of a Standard Operating Procedure for determination of sulfur mustard protein adducts; histidine adducts

Although histidine adduct are the most abundant adducts resulting after exposure of proteins to sulfur mustard, there is no sensitive method for their analysis available. For the histidine adducts a convenient isolation procedure from acidic hydrolysates was developed based on cation exchange chromatography. Furthermore, a derivatization method for GC-MS analysis was developed, which allows the analysis of the histidine adducts as tris(trifluoroacetyl)derivatives. The detection limit for GC-MS analysis of this compound is 3 ng/ml; 3 pg absolute.
Attempts to analyze the histidine adduct as a hydrazide, resulting from treatment of globin with hydrazine, failed mainly due to the instability of the hydrazide functionality.

Derivatization of the histidine adduct with pentafluorobenzyl bromide resulted in the formation of the (tris)pentafluorobenzyl derivative. This derivative could not be analyzed by means of GC-MS, probably because of the charged imidazole ring. However, it turned out that it has favorable properties for LC tandem MS analysis. It has a long retention time, compared to other derivatives, which might enable Sep-pak clean-up of real samples containing the particular adduct. In the next grant period it will be investigated whether the tris(PFB) derivatives can also be analyzed under electron capture APCI conditions. The latter technique has recently been reported to give improved detection limits.

We also proposed to study the feasibility of fluorescence detection of sulfur mustard adducts. Although HPLC with fluorescence detection is much less specific than mass spectrometric detection, it has the advantage that yoctomole amounts of amino acid derivatives can be analyzed. In the current grant period a number of fluorescent derivatives of the histidine-adduct were synthesized, which will be used as reference compounds. No problems were encountered thus far. In the next year of the grant period, the derivatization of processed acidic digests will be studied, as well as the detection limits that can be obtained.

Immunochemical protein adduct analysis

The main advantage of detection of adducts to proteins over those to DNA is the expected much longer half-life of the protein adducts. Therefore, antibodies were raised against S-HETE-cysteine in partial sequences of human hemoglobin in our previous studies (*4*). However, the minimum detectable concentration obtained for *in vitro* exposure of human blood with these antibodies was only 50 μM of sulfur mustard. Consequently, further exploratory research on immunochemical assays of protein adducts was a major topic of the current study.
Investigations were performed on three proteins, *i.e.*, hemoglobin, albumin, and keratin. The accessibility of the adducts for immunochemical analysis is supposed to increase in this order, *i.e.*, hemoglobin is enclosed in erythrocytes, albumin is freely circulating in the plasma, whereas keratins in the skin are directly accessible from the environment for sulfur mustard and for reagents.

Immunochemical analysis of hemoglobin adducts; characterization of monoclonal antibodies against cysteine-sulfur mustard adducts in hemoglobin
Antibodies (clone 3H6) raised against an alkylated peptide, *i.e.*, N-acetyl-S-HETE-cys_{93} through leu_{106}-lys of the β-chain of hemoglobin, were further characterized. It appeared that exposure of human hemoglobin to 50 μM sulfur mustard was detectable in a direct ELISA. However, the direct ELISA is usually not the most sensitive immunochemical assay. Therefore, we have attempted to apply these antibodies in an immunoslotblot assay to alkylated hemoglobin. Several other clones were obtained from the above-mentioned

immunization which produced antibodies that recognize alkylated hemoglobin, but have still to be screened for their efficacy to bind to sulfur mustard adducts to globin.

Immunochemical analysis of hemoglobin adducts: antibodies against peptide haptens containing a histidine-sulfur mustard adduct
N1/N3-HETE-Histidine is the most abundant amino acid adduct formed in hemoglobin (and albumin, *vide infra*) after exposure of human blood to sulfur mustard (*4*). In addition, three out of the five sites of alkylation within the tertiary structure of hemoglobin are histidine residues, *i.e.*, α-his_{20}, β-his_{77} and β-his_{97} (*5*). Therefore, partial sequences of hemoglobin containing these adducted amino acids were synthesized as haptens and used for raising antibodies (*12*).

From all haptens clones were obtained producing antibodies with specificity for hemoglobin treated with 50 µM sulfur mustard. For the screening of these clones in order to select the most effective antibodies the test procedure should be optimized first. It appeared that coating of the plates with 10-12.5 µg/ml globin and applying a rather high dilution of 2nd ab (1:1000 or 1:2000) resulted in the highest specificity for the detection of sulfur mustard adducts to globin. These data suggest that the immuno assay for screening of monoclonals producing ab's against sulfur mustard adducts to globin can still further be improved by suppressing the a-specific binding of 1st ab to globin, for example by carrying out the 1st ab adsorption at a higher concentration of Tween 2. This optimized test system will now be used for the screening.

Immunochemical analysis of albumin adducts
An electrophilic compound has to cross the cell membrane of the erythrocyte in order to react with hemoglobin. Therefore, adduct formation with plasma proteins might be more efficient. The most abundant plasma protein is albumin, which has a relatively slow turn-over in human beings (half-life 20-25 days). It was found that a proportional amount of sulfur mustard (approximately 20%) was bound to albumin isolated from human blood treated with sulfur mustard (*12*).

We attempted to raise antibodies against the synthesized T5 fragment (*vide supra*) of human albumin containing an alkylated cysteine and against the alkylated human serum albumin itself. Unfortunately, all originally positive clones lost their specificity for alkylated human albumin during the selection process. These data suggest that the immunogenicity of the HETE-moiety as present in this peptide is not very high and that possibly the alkylated T5-sequence in the sulfur mustard-exposed albumin is poorly accessible.
Antibodies raised against the other alkylated proteins (hemoglobin and keratin) did not show any cross-reactivity with sulfur mustard adducts to albumin.

Immunochemical analysis of keratin adducts
In addition to the respiratory tract, the skin is a major target for vesicants such as sulfur mustard. Proteins in the skin, particularly those in the stratum corneum, are readily accessible to agents. Since keratin is the most abundant protein in stratum corneum and epidermis, methods for retrospective detection of skin exposure to sulfur mustard were developed in the present study.
Keratins (MW 40-70 kDa) form the backbone of the intermediate filaments (IFs) in epithelial tissues (*26, 27*). In basal epidermal cells almost 30% of all synthesized proteins are keratins. Their structures are closely related and can be represented by a central α-helix rich domain (length 300-350 residues) flanked on either side by non-helical domains of variable size and chemical character. The helical segments contain heptad repeats of hydrophobic residues. In addition, a conserved periodic distribution of acidic (aspartic acid, glutamic acid) and basic (arginine, histidine and lysine) amino acids is found in IFs. The termini contain *inter alia* inexact repeats of glycine and (phospho)serine residues. Amino acid sequences of a number

of human keratins have been documented (*26*). Most data indicate that the end domains are predominantly located on the surface of the IFs.

For development of an immunochemical assay, two partial end domain sequences of keratin K14 and one partial end domain sequence of keratin K5 were synthesized on a solid support as haptens for raising antibodies, as earlier described (*12*). This approach appeared to be very successful for raising antibodies (*15*). Fusions from mice immunized with only one peptide or with a mixture of two or three peptides resulted in specific antibodies to sulfur mustard adducts in keratin isolated from human callus. Antibody 1H10 was raised against adducts present in end domains of keratins K5, K14 and K1, of which K5 and K14 are two of the most important keratins in basal cells and K1 is the main keratin in the stratum corneum. The competitive ELISA turned out that antibody 1H10 only recognised sulfur mustard adducts to peptide 2 which contained amino acid sequences present both in end domains of K1 and K5.
Furthermore it appeared also important to apply freshly prepared supernatants of cultures of clones producing specific antibodies.
The preliminary data with the immunoslotblot assay suggest that a lower detection limit of 0.2 μM sulfur mustard exposure should be feasible (or 0.3 s exposure to saturated sulfur mustard vapor). As can be derived from the binding data of [^{14}C]sulfur mustard to keratin, presented previously (*12*), this corresponds to 25 fmol of adducted sulfur mustard at 0.5 μg of keratin blotted to the nitrocellulose filter, and to 1 sulfur mustard adduct among $5x10^7$ unadducted amino acids in keratin, or 1 sulfur mustard adduct per 10^5 keratin molecules.

Cross-reactivity of antibodies for sulfur mustard adducts with hemoglobin, albumin and keratin
Cross-reactivity was observed for antibodies raised against partial sequences of hemoglobin containing a histidine-sulfur mustard adduct. These antibodies show specificity not only for alkylated hemoglobin but also for alkylated keratin. This suggests that the specificity depends in some cases mainly on the presence of the 2-hydroxyethylthioethyl moiety and not on the amino acid to which this moiety is bound. If so, these antibodies can be valuable for immunochemical staining applications, *e.g.*, in histochemical identification of alkylated cell proteins in human skin.

These antibodies did not show specificity to albumin or trypsinized albumin alkylated by sulfur mustard. Similar negative results were obtained for one of the clones raised against a partial sequence of keratin containing a glutamine-sulfur mustard adduct, which showed a positive response on sulfur mustard-exposed human skin. We were also unsuccessful in raising antibodies against sulfur mustard treated albumin when using a partial sequence of albumin containing the major adduct formed in this protein with sulfur mustard (cysteine-34, *vide supra*). These results may suggest that sulfur mustard adducts in albumin are poorly accessible.

VI KEY RESEARCH ACCOMPLISHMENTS

1. The alkylated cysteine-34 residue in albumin is a highly sensitive biomarker for exposure to sulfur mustard.
2. A novel, rapid isolation procedure for albumin, based on affinity chromatography, has been introduced.
3. The lowest detectable exposure level in human blood for determination of the alkylated cysteine-34 residue in albumin has been improved with a factor of 10, by increasing the amount of albumin.
4. The particular albumin - sulfur mustard adduct is stable in the marmoset in vivo, at least 28 days after the exposure.
5. A convenient isolation procedure for the histidine adducts has been developed, based on cation-exchange chromatography.
6. A GC-MS derivatization reaction for the histidine adducts has been developed, which enables sensitive detection of the adduct.
7. Several fluorescent derivatives of the histidine adducts have been prepared which might enable highly sensitive analysis by means of laser-induced fluorescence.
8. On the basis of the present results, it can be expected that Standard Operating Procedures for diagnosis of exposure to low levels of sulfur mustard will become available in the near future.
9. Several clones are available producing antibodies which show specificity not only for hemoglobin alkylated with 50 μM sulfur mustard but also for alkylated keratin.
10. An improved screening procedure has been set up for selection of antibodies from clones raised against adducted peptides with amino acid sequences present in human globin.
11. An immunoslotblot assay was set up for detection of sulfur mustard adducts to keratin.
12. Up to now, the lower detection limit of the immunoslotblot assay for detection of sulfur mustard adducts to keratin was at 25 fmol adducted sulfur mustard using 0.5 μg keratin/slot, corresponding to an adduct level of 1 sulfur mustard adduct/ $5x10^7$ unadducted amino acids. The minimum detectable concentration for *in vitro* treatment of human callus with sulfur mustard was 0.2 μM.

VII REPORTED OUTCOMES

Publications

NOORT, D., FIDDER, A., HULST, A.G., AND LANGENBERG, J.P. (2002) Low level exposure to sulfur mustard: development of an SOP for analysis of albumin and/or hemoglobin adducts. Proceedings of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

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VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., AND LANGENBERG, J.P. (2002) Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Proceedings of the meeting of NATO TG-004, November 2002, Oslo, Norway.

Abstracts

NOORT, D., FIDDER, A., HULST, A.G., AND LANGENBERG, J.P. (2002) Low level exposure to sulfur mustard: development of an SOP for analysis of albumin and/or hemoglobin adducts. Book of abstracts of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

VAN DER SCHANS, G.P., NOORT, D., MARS-GROENENDIJK, R.H., FIDDER, A. AND LANGENBERG, J.P. (2002) Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. Book of abstracts of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., AND LANGENBERG, J.P. (2002) Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Proceedings of the meeting of NATO TG-004, November 2002, Oslo, Norway.

Presentations

Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Presented by G.P. Van der Schans at meeting of NATO TG-004, November 2002, Oslo, Norway.

VIII CONCLUSIONS

The need for retrospective detection procedures for exposure to low levels of chemical warfare agents has been urgently illustrated by the conflicts in the Gulf Area and, especially, in the attempts to clarify the Gulf War Syndrome. Explorative research within the context of our previous grant DAMD17-97-2-7002 has yielded important clues for development of standard operating procedures that satisfy these needs. The aim of the present research is twofold:

1. Development of a mass spectrometric or fluorescence-based method for retrospective detection of exposure to low doses of sulfur mustard, based on improvement of analysis of an adducted tripeptide in albumin and of adducts to histidine in hemoglobin and albumin.
2. Development of sensitive immunoslotblot assays for quantitation of protein – sulfur mustard adducts.

The albumin assay is based on our previous finding that upon pronase treatment of sulfur mustard alkylated albumin, a tripeptide cysteine(S-2-hydroxyethylthioethyl)-proline-phenylalanine ((S-HETE)Cys-Pro-Phe) results which has favorable mass spectrometric properties. The procedure for isolation of albumin from human blood could be substantially shortened by performing the incubation with $CaCl_2$ for only 1 h, instead of overnight. Moreover, an affinity chromatography procedure was developed which enables work-up, digestion and mass spectrometric analysis of a plasma sample within 3 hours.
The lowest detectable exposure level was improved by one order of magnitude (1 nM) by work-up of larger amounts of albumin. Furthermore, the use of an internal standard, *i.e.*, albumin isolated from human blood exposed to d_8-sulfur mustard, has been worked out. This will enable quantitative analyses of unknown samples.

Derivatization of (S-HETE)Cys-Pro-Phe with pentafluorobenzyl bromide (PFB-Br) in the presence of KOH in acetonitril afforded the di-PFB derivative, which enabled mass spectrometric detection under Electron Capture Atmospheric Pressure Chemical Ionization (EC APCI) conditions.

With respect to forthcoming animal experiments, in which rats will be exposed to sulfur mustard in order to obtain information about the persistence of the albumin-sulfur mustard adduct, the level of alkylation at the free cysteine residue in rat albumin was determined to be 5%. After pronase treatment of rat albumin, isolated from rat blood exposed to [^{14}C]-labelled sulfur mustard, the tripeptide Cys(HETE)-Pro-Tyr could be analyzed in an analogous way as performed for (S-HETE)Cys-Pro-Phe.
In albumin isolated from plasma samples taken from marmosets which had been challenged (i.v.) with sulfur mustard within the context of our previous cooperative agreement, the tripeptide (S-HETE)Cys-Pro-Phe could still be analyzed after 28 days. Taken into account that the mass spectrometer (Q-TOF) used for these experiments is not the most sensitive one for this kind of analyses, the retrospectivity for this particular adduct is high.

With regard to analysis of the histidine-sulfur mustard adducts (N1- and N3-isomers, *i.e.*, the most abundant amino acid adducts formed after exposure to sulfur mustard), a work-up procedure was developed for rapid isolation of these adducts from amino acid mixtures resulting from acidic hydrolysis of globin or albumin. This procedure consists of an ion chromatography step by using Dowex 50WX8 (Na^+ form). Uncharged amino acids were eluted with aqueous acetic acid. Subsequent elution with diluted NH_4OH afforded highly purified histidine adducts, which could be further derivatized with Fmoc-Cl for electrospray LC tandem MS analysis, or with fluorescent labels for detection with laser induced fluorescence.

From our previous research it appeared that GC-MS based procedures for analysis of the histidine adducts were troublesome, mainly due to the high polarity or thermal instability of the derivatives. We have now found that derivatization with trifluoroacetic anhydride results in the formation of a (tris)trifluoroacetyl derivative which has favorable GC-MS properties. Derivatization of the histidine adducts with pentafluorobenzyl bromide in DMF, in the presence of triethylamine, afforded the corresponding tris-(pentafluorobenzyl) derivatives. The derivative exhibited favorable properties for LC-tandem MS analysis.

For detection with laser-induced fluorescence, various derivatization methods have been explored and several fluorescent derivatives of the histidine adducts have been prepared.

Within the framework of previous grants we have raised antibodies directed against adducts of sulfur mustard with proteins. In the current grant we have developed an improved screening procedure for the selection of monoclonal antibodies against adducts of sulfur mustard with proteins (globin, albumin and keratin). The most suitable antibodies are used for the development of an immunoslotblot assay for retrospective detection of exposure to sulfur mustard.

The direct ELISA applied for screening of antibodies on their efficacy to bind to adducted proteins appeared to be not sufficiently reproducible. Therefore much effort was spent to improve the assay. It appeared that enhancement of the antigen concentration to 10-12.5 μg/ml resulted in optimal presentation of sulfur mustard adducts on the protein. In addition, a further dilution of 2^{nd} antibodies and limited adsorption times (30 min) for both 1^{st} antibodies and 2^{nd} antibodies provided a better specific/a-specific ratio of fluorescence signal.

The fact that freshly prepared supernatants of cultures of clones contained much higher antibody activities than supernatants stored in the freezer (for up to 2 years) indicated that storage under frozen conditions resulted in a slow loss of activity. This means that the use of only recently prepared supernatants is recommended unless better storage conditions can be applied.

Upon characterization in a competitive ELISA one of the antibodies, raised against peptides derived from end domains of keratin K5 and K14, recognized specifically a sulfur mustard adduct on a amino acid sequence both present in keratin K5 and K1, but not that on a sequence present on an end domain of keratin K14. On the other hand, the same antibodies recognized also to some extent adducts to another peptide derived from keratin 14.

The immunoslotblot assay under development is a modification of that developed for the detection of N7-HETE-Gua in DNA, described previously (*12*). So far the best results are obtained with the detection of sulfur mustard adducts to keratin isolated from human callus exposed to sulfur mustard. Up to now, the lower detection limit of the immunoslotblot assay for detection of sulfur mustard adducts to keratin was reached at 25 fmol adducted sulfur mustard using 0.5 μg keratin/slot, corresponding to an adduct level of 1 sulfur mustard adduct/ $5x10^7$ unadducted amino acids. The minimum detectable concentration for *in vitro* treatment of human callus with sulfur mustard was 0.2 μM.

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XI LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE AGREEMENT

Dr. D. Noort
Dr. G.P. van der Schans
Dr. B.L.M. van Baar
Mr. A. Fidder
Mrs. R.H. Mars-Groenendijk
Mr. A.G. Hulst
Mrs. S. de Kant
Mrs. C.E.A.M. Degenhardt-Langelaan
Mr. E. Wils
Mr. S.H. van Krimpen